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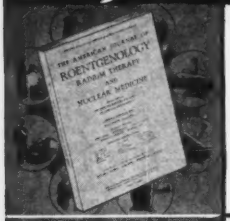

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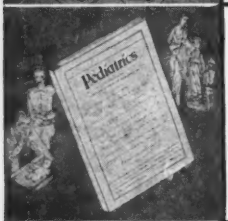

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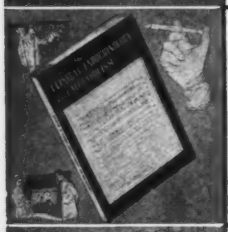

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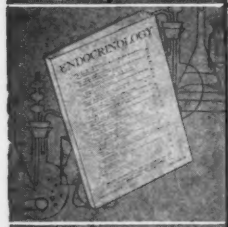

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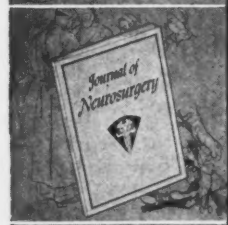

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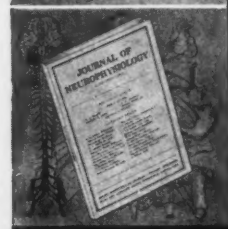

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NOTICE TO CONTRIBUTORS

A change in the format of ENDOCRINOLOGY will go into effect July 1, 1956. Manuscripts submitted after February 1, 1956 should conform to the new specifications for the preparation of manuscripts as printed in this issue (pages ii and iv).

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SEXUAL BEHAVIOR IN CASTRATED-ADRENALECTOMIZED HAMSTERS MAINTAINED ON DCA[1]

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INTRODUCTION

THE persistence of many components of sexual behavior for a considerable time after castration is commonly observed in male individuals of many vertebrate species (Stone, 1927, 1932; Beach, 1944). An explanation frequently advanced is that in castrated (or gonadless) males extratesticular androgen from the adrenal cortex is responsible for the continuing sexual activity (Sollenberger and Hamilton, 1939; Beach, 1948:25; Morgan and Stellar, 1950:426; Dempsey, 1951:216; Kinsey *et al.*, 1953: 755). Until now this hypothesis has not been tested experimentally except for preliminary reports by Pauker-Warren and Aronson (1952) and by Schwartz and Beach (1954). The present study was directed toward revealing the possible influence of the adrenal cortex on sexual behavior; in particular, to test the hypothesis that adrenal cortical secretions are responsible, at least in part, for the persistence of sexual behavior after castration. Since morphological changes in the seminal vesicles are frequently used as indicators of androgen level, changes in the histology of these glands were correlated with the behavioral data.

The golden hamster was selected as an appropriate experimental animal for three major reasons. First, the hamster demonstrates a considerable amount of sexual activity after gonadectomy. Second, after castra-

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[1] This study is based on one section of a dissertation by the first author to the Faculty of the Graduate School of Arts and Science of New York University in partial fulfillment of the requirements for the degree of Doctor of Philosophy. It was supported in part by a grant from the Committee for Research in Problems of Sex, National Research Council.

The authors wish to express their appreciation to Dr. T. C. Schneirla for critically reading the manuscript and to Mr. Elwood Logan, of the Museum Photography Division for the photomicrographs.

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tion the seminal vesicle epithelium is reported to present a more or less normal appearance for about two months, which suggested to Ortiz (1953) a possible androgenic influence of the adrenal gland. Third, we were unable to identify any accessory adrenal cortical tissue in preliminary experiments.[3]

MATERIALS AND METHODS

The hamsters used in this experiment were purchased from a dealer or were born and reared in the laboratory. Care and housing of the animals were essentially similar to that described in a previous paper (Pauker, 1948). Sex tests of males were run with naturally receptive females or females brought into heat with estrogen-progesterone injections. The testing procedure was similar to that described previously (Pauker, 1948) except that in the present study the tests were run for 10 minutes rather than fifteen. Basic features of the mating pattern that have been quantified are mounting, copulation (i.e. intromission with or without ejaculation), and copulatory latency time (i.e. the interval between the introduction of the female into the testing chamber and the occurrence of the first copulation). A minimum of five preoperative tests were conducted on each mature male hamster, beginning at approximately seven to nine weeks of age. The preoperative tests were never spaced less than four days nor more than 21 days apart. These variable intervals had no noticeable effect on the behavior during the sex test (Beach and Pauker, 1949). Since there is considerable variation among individuals with respect to sexual performance, the animals were divided into the experimental groups after the preoperative tests were completed. Thus the groups were equalized so that the average behavior for each group was similar with regard to frequency of mounting, copulation, and copulatory latency time ($P > .05$).

The following four groups of animals were studied:

Group I. *Castrated untreated males*—10 animals.

Group II. *Castrated males given DCA.*

a. Four animals received daily injections of 1.0 mg. DCA[4] in 0.1 cc. of sesame oil for eight weeks beginning on the day of castration. After eight weeks the dose was increased to 2.0 mg./0.2 cc. oil daily for one week, followed by a final week in which 3.0 mg./0.3 cc. oil was administered daily. Throughout the DCA treatment, the animals were tested weekly for 10 weeks postoperatively.

b. In each of five animals a DCA pellet weighing approximately 40 mg. was implanted subcutaneously at the time of castration.

Group III. *Adrenalectomized males given DCA.*

a. Four animals received daily injections of 1.0 mg. DCA in 0.1 cc. of sesame oil for ten weeks, beginning at the time of adrenalectomy. The animals were tested weekly throughout this period.

b. In each of thirteen animals a DCA pellet weighing approximately 40 mg. was implanted subcutaneously at the time of adrenalectomy.

[3] Kent and Chapman (1951) obtained 70% to 100% survival of hamsters after a two stage adrenalectomy, a finding which they attribute to substantial accessory adrenal cortical tissue. These contradictory results may reflect strain differences, or possibly operative techniques. We believe that it is extremely important to remove the periadrenal fat along with the glands to insure that the capsule of the gland remains intact.

[4] The DCA, Cortate, was kindly furnished by the Schering Corporation, Bloomfield, New Jersey.

Group IV. *Castrated-adrenalectomized males given DCA.*

Eighteen animals were castrated. In seven of these a DCA pellet weighing approximately 40 mg. was implanted subcutaneously in the abdominal region. Two or three days later they were adrenalectomized. In each of the remaining eleven males, a DCA pellet was implanted subcutaneously on the dorsum at the time of adrenalectomy.

Operations were performed under nembutal anesthesia, supplemented with ether when necessary. Animals were castrated through the abdominal approach. Adrenalectomies were performed bilaterally in a one-stage operation. A large area of surrounding fat was removed with the gland.

All animals were tested weekly for sexual behavior for at least six weeks after operative procedures. Some animals were tested for longer periods (see results). The data were analyzed by means of the analysis of variance and Student "t" tests.

When the postoperative testing schedule was completed, the castrated untreated and the castrated-DCA treated animals were killed with ether and the seminal vesicles were removed and fixed in Bouin's solution. The adrenalectomized and castrated-adrenalectomized males were taken off DCA therapy (i.e., the injections were stopped or the pellet removed under light ether anesthesia), and the animals were watched for insufficiency symptoms. The adrenalectomies were considered to have been complete if the animals died following hormone withdrawal. All fatalities occurred within 12 days. Since tissues taken at death would not be representative of the seminal vesicles in the well maintained animal, two adrenalectomized males and two adrenalectomized-castrated males were subjected to seminal vesicle biopsies before DCA withdrawal. In the males which were only adrenalectomized one testis from each was also removed. The animals were allowed to recover for at least a week whereupon the hormone was withdrawn and the animals allowed to die of adrenal insufficiency. Autopsies were performed in all cases as a further check on the completeness of adrenal removal. The seminal vesicles and testes were imbedded in paraffin, sectioned at 8μ and stained with Harris's hematoxylin and eosin. The epithelium of the seminal vesicles of the four groups was studied and measured with an ocular micrometer.

RESULTS

Survival. All animals in Groups I and II (the castrated and castrated-DCA treated animals) survived the course of the experiment. Of 17 operated animals in Group III (adrenalectomized+DCA males) eleven survived the entire experiment. All but one of these succumbed within twelve days after DCA therapy was withdrawn. The one survivor was found to have regenerated adrenal cortical tissue in the right perirenal fat area. The data on this animal are not included in the results. Of 18 males in Group IV (castrated-adrenalectomized males on DCA) 11 remained in good condition throughout the experiment. All of these animals succumbed within two weeks after cessation of hormone therapy.

Number of males achieving copulation. In Groups I, II and IV in which castration was included in the operative procedure, a progressive decrease in the number of animals copulating occurred by the third postoperative week (Fig. 1). The rate of decrease in the second to sixth postoperative tests was essentially the same in each group, and group differences in the percentages of animals achieving copulation per test proved to be insignificant. In contrast to this decline for Groups I, II and IV, the average

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number of males copulating in Group III (adrenalectomized and given DCA but not castrated) remained the same throughout the experiment.

In the first postoperative test, the number of castrated-adrenalectomized animals (Group IV) that copulated was considerably less than for the other groups. We believe that the animals in this group were still suffering from the traumatic effects of adrenalectomy at the time this test was conducted.

Frequency of copulation. In the first postoperative test the average frequency of copulation in Group IV (castrated-adrenalectomized) fell to a

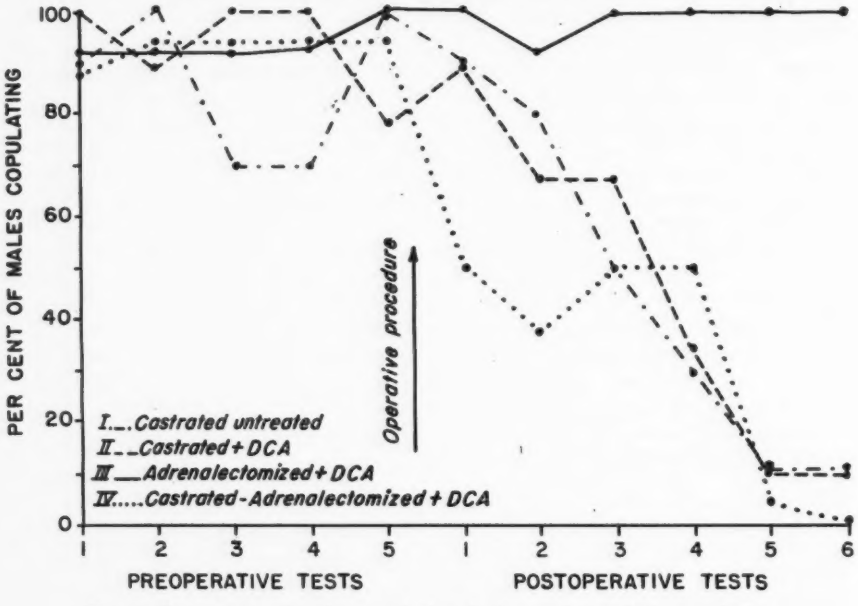


FIG. 1. Percentage of males copulating during each test before and after operative procedures.

very low level (Fig. 2). Fewer animals achieved copulation, and among those that did, one half had frequencies below their preoperative average. As was explained earlier, these animals were tested only four days after adrenalectomy. However, during the second postoperative test, the copulatory frequency of Groups I and II, which had remained high at the end of the first week after castration, fell sharply to a level comparable to Group IV whose frequency was essentially unchanged. Thus at the second testing period after operation the differences in frequency of copulation among Groups I, II and IV were not significantly different at the .05 level. In the following weekly tests, Groups I, II and IV showed a similar progressive decrease in frequency of copulation and no statistical difference among them could be demonstrated. These groups now had significantly lower

copulatory frequencies than the adrenalectomized animals with intact gonads (Group III).

Number of males mounting. Mountings unaccompanied by complete intromissions occurred in all tests in which copulations were achieved (Fig. 3). Also mountings were frequent in tests in which intromissions did not occur. A rather high proportion of males in all groups mounted during the first four postoperative tests. During the fifth test, the number of males that mounted decreased to a similar extent in Groups I, II and IV,

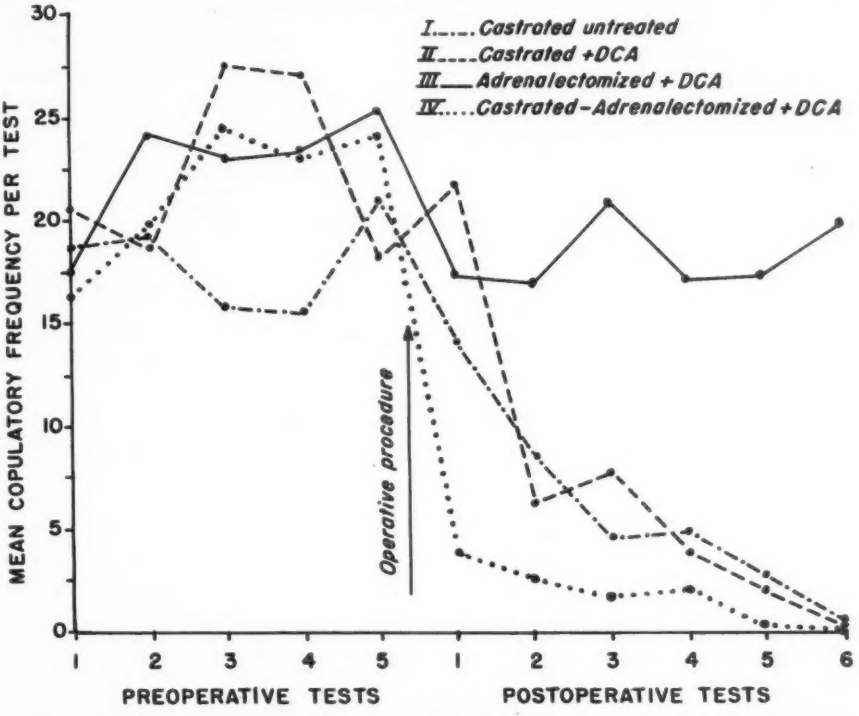


FIG. 2. Mean copulatory frequency per test before and after operative procedures.

while all animals in Group III continued to mount. On the sixth postoperative test, 50%, 40% and 55% of the animals in Groups I, II and IV respectively were still mounting.

Mounting frequency. In the first postoperative test the mounting frequency of Group IV was significantly higher than in the other groups (Fig. 4). In the second, third and fourth postoperative tests, there was no significant difference in the mounting frequency among the four groups. However by the fifth postoperative test, the mounting frequency of the castrated and castrated-DCA animals dropped significantly below that of the adrenalectomized males. In the sixth test all three castrate groups

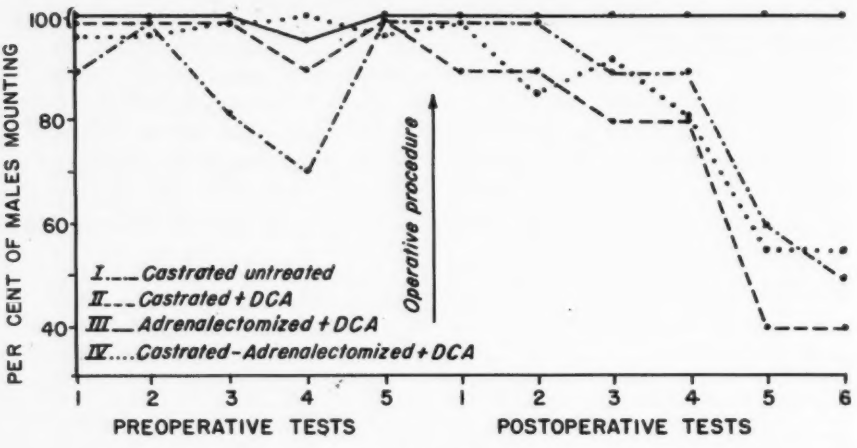


FIG. 3. Percentage of males mounting during each test before and after operative procedures.

had significantly lower scores than that of the adrenalectomized group. The mounting frequency of the castrated-adrenalectomized group never dropped significantly below that of castrate Group I during any of the postoperative tests.

When preoperative tests 1–5 of Group III were compared with postoperative tests 1–5 we found a significant increase in mounting activity

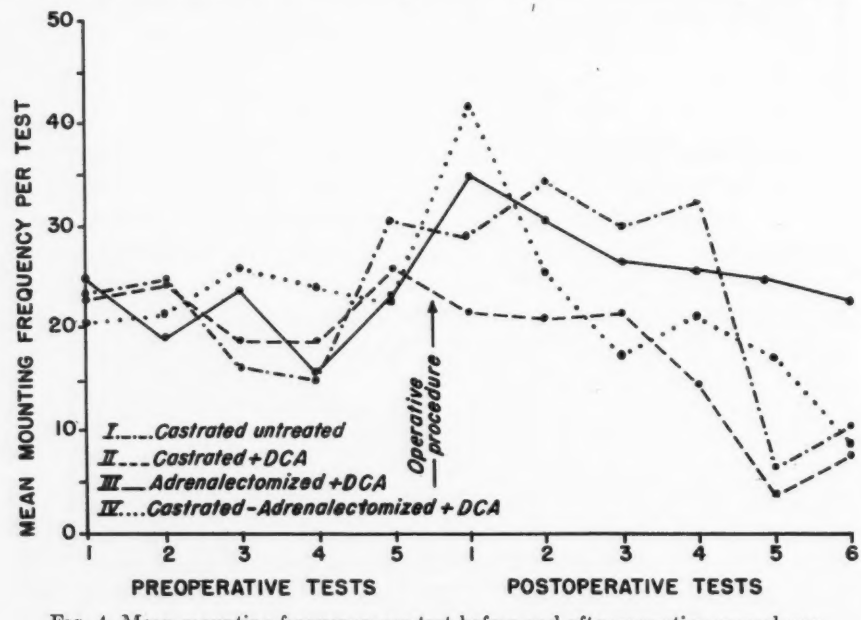


FIG. 4. Mean mounting frequency per test before and after operative procedures.

(Mean diff. = 41.1, $P < .01$). This is similar to the observations of Beach and Pauker (1949) that mounting behavior (called "abortive attempts") in normal hamsters increases in successive tests up to a certain level. In other words, adrenalectomy+DCA did not upset the expected trend of increasing mounting activity.

Copulatory latency time. In those animals able to execute a complete intromission after castration, castration+DCA, and castration-adrenalectomy+DCA, the latency period was not appreciably affected. Adrenalectomy alone had no significant effect on copulatory latency time.

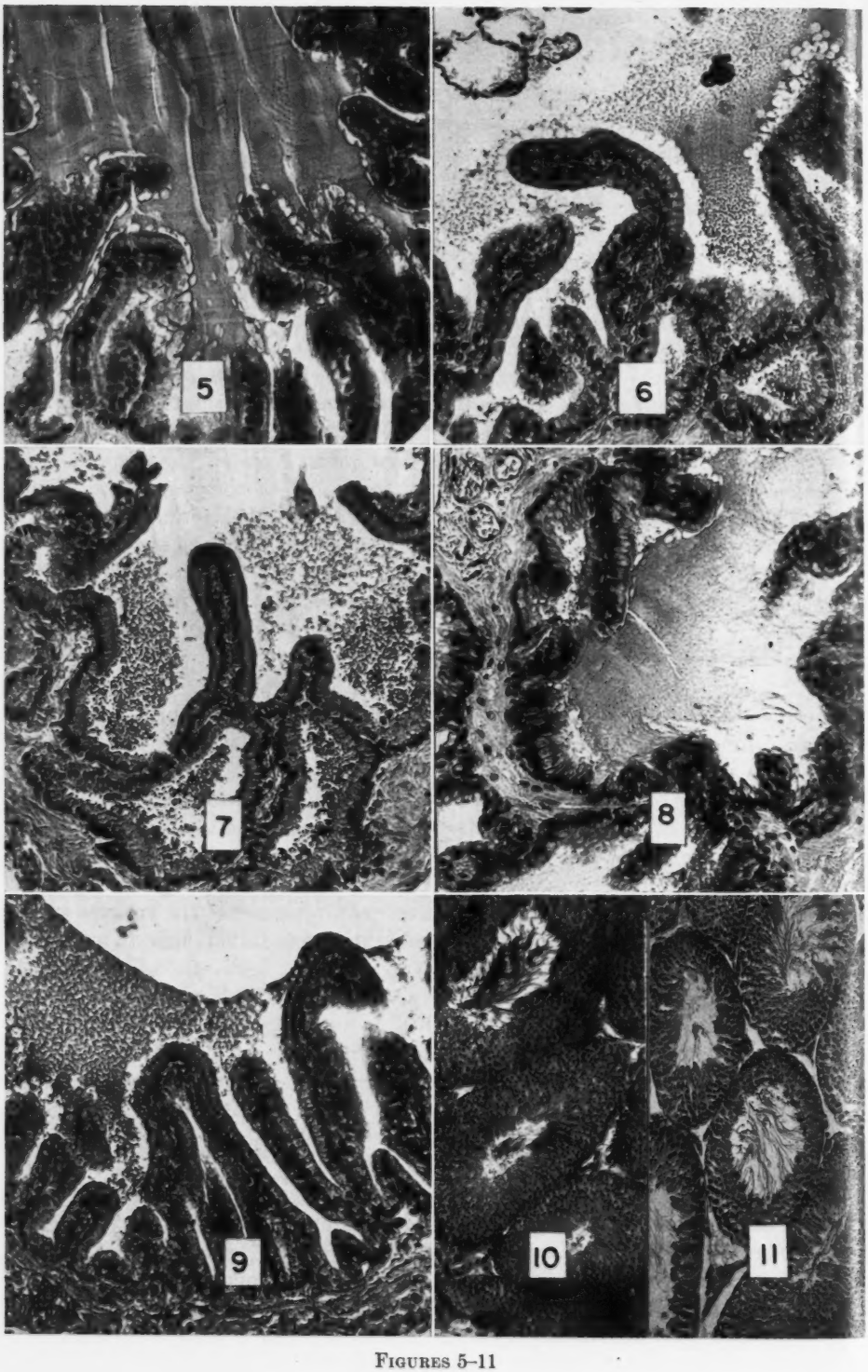
Males tested for ten weeks after operative procedures. As a further test for possible influences of DCA on sexual behavior, four animals of the castrated+DCA group that had received subcutaneous injections of 1 mg. of DCA daily for eight weeks postoperatively, were now given 2 mg. daily for one week, then 3 mg. daily for a final week. None of these animals showed any increase in sexual activity from that of the castrate level.

Three of the four adrenalectomized males of Group III that received subcutaneous injections of 1 mg. of DCA daily for 10 weeks were maintained in good condition throughout the experiment. These adrenalectomized animals exhibited sexual behavior comparable to their preoperative, pre-DCA levels. Injections were stopped during the 11th week after adrenalectomy and the animals were tested again five days after cessation of hormone therapy. Two of the three animals copulated and sexual activity still appeared normal although symptoms of adrenal insufficiency were pronounced. Within two to three days after this last test, the three animals were dead.

The effect of castration and castration+DCA on the seminal vesicles. The mean weights of the seminal vesicles were reduced to about half by castration. Although the vesicular epithelium was not as strikingly affected, definite degenerative signs were observed. Moreover DCA treatment from the time of castration did not prevent the loss in weight of the vesicles or the degenerative changes in the epithelium. In the intact male hamster the epithelial cells range from cuboidal to columnar between 18μ and 33μ in height (Fig. 5). Although many areas were found within this range in the castrated males with or without DCA treatment (Figs. 6 and 7), areas of local degeneration were also evident. In these regions cytoplasmic threads extruded from the distal end of the cell into the lumen of the vesicles. Signs of secretion were present in the lumen, but the products were scant and presented a granular appearance rather than the homogeneous aspect of the normal seminal fluid.

The effect of castration-adrenalectomy+DCA on the seminal vesicles. Adrenalectomy coupled with castration had no further degenerative effects on the seminal vesicles than castration alone (Fig. 9). Both the macroscopic and microscopic pictures of the vesicles were similar to those of the castrated and castrated-DCA treated animals.

The effect of adrenalectomy+DCA on the testes and seminal vesicles.



FIGURES 5–11

Adrenalectomized adult hamsters maintained in good health on DCA had testes comparable in size to those of intact males. The structure of the testes appeared normal. Leydig cells were typically distributed. Active spermatogenesis was occurring in the seminiferous tubules and the lumens were full of sperm tails (Figs. 10 and 11). Final evidence that the testes were in an active physiological condition was the ability of these males to achieve repeated fertile matings with estrus females. The seminal vesicles appeared normal and active secretion was observed (Fig. 8).

DISCUSSION

The results of preliminary experiments together with the findings of Snyder and Wyman (1951) demonstrated that adrenalectomized hamsters could not be maintained on NaCl but could be kept in good health on DCA. Since the purpose of this experiment was to study the effect of adrenalectomy on sexual behavior in the male hamster with intact testes and after castration, it became necessary to determine whether DCA had any andromimetic effect on sexual activity.

Is DCA Androgenic? DCA in the quantities used in this experiment had no effect on the sexual behavior of the castrated male hamster, even when the dose was increased to three times that used to maintain adrenalectomized hamsters in good health. That is, the decline of copulatory ability which usually follows castration was not prevented. Furthermore, DCA did not affect the size or the histological picture of the seminal vesicles in the castrates.

Numerous other investigators have concluded that DCA is not androgenic with regard to its effect on tissues (Green and Burrill, 1940; Selye and Albert, 1942; Howard, 1946; Robson, 1951), although a report to the contrary by Hooker and Collins (1940) is frequently cited. These authors reported that mice and rats injected with DCA for several days following castration resulted in a significant maintenance of the seminal vesicles and prostate gland. The difference in size of the seminal vesicle between un-

EXPLANATION OF FIGURES 5–11

FIG. 5. Seminal vesicle of normal adult male. H. & E., ×200.

FIG. 6. Seminal vesicle 6 weeks after castration. H. & E., ×200.

FIG. 7. Seminal vesicle 6 weeks after castration and implantation of 40 mg. pellet of DCA. H. & E., ×200.

FIG. 8. Seminal vesicle 6 weeks after adrenalectomy and implantation of 40 mg. pellet of DCA. H. & E., ×200.

FIG. 9. Seminal vesicles 6 weeks after castration plus adrenalectomy and implantation of 40 mg. pellet of DCA. H. & E., ×200.

FIG. 10. Testis of normal adult male. H. & E., ×100.

FIG. 11. Testis 6 weeks after adrenalectomy and implantation of 40 mg. pellet of DCA. H. & E., ×100.

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treated and DCA treated castrated rats and mice was their sole criterion for andromimetic function of DCA. However, the samples used were so small (in the case of the rats two experimental and one control) that differences in size of the gland could still be within a normal population variability. Further, the authors were unable to demonstrate that DCA had any stimulating effect on the seminal vesicle epithelium.

Adrenalectomy. Removing the adrenals of adult male hamsters and maintaining these animals in a healthy condition with DCA had no effect on the gonads, reproductive behavior, or the ability to successfully impregnate females.

Claims have been made by some investigators that adrenalectomy in adult animals has a direct inhibitory effect on the testes (Jaffe, 1923; Freed, Brownfield and Evans, 1931; Fitzhugh, 1937). On the other hand Gaunt (1933) and Moore (1953) were unable to find any regression of the testes following adrenalectomy in the rat. Animals suffering from adrenal insufficiency, that are not properly maintained on some therapeutic regimen such as DCA, are likely to exhibit degenerative changes in the reproductive system and loss of "libido" as reported by Selye (1947). However, these effects can not be considered specific, since the entire organism is in a state of progressive debilitation.

Castration-adrenalectomy. It is generally agreed that under certain pathological conditions the adrenal cortex is capable of elaborating physiological quantities of a gonad-like hormone. Likewise, certain strains of laboratory animals when castrated develop adenomas of the adrenal cortex that elaborate sex hormones in such quantities as to reestablish the integrity of the existing reproductive structures and sexual behavior. Since castration of all male vertebrates that have been studied results in an increase in the size of the whole adrenal cortex or of specific zones, and since there appears to be a similarity between adrenal steroids and the testicular steroids, a capacity of the adrenal gland to elaborate increased quantities of "sex hormones" after gonadectomy has been postulated (Botella Llusia, 1952). These observations have led investigators to suggest that even in animals which do not develop extreme pathology after castration, cortical secretions may be sufficient to activitate to some extent the mechanisms responsible for the observed reproductive behavior. However, the present study has demonstrated that in the hamster at least the adrenal gland is not necessary for the residual sexual activity after castration.

It has been suggested that the seminal vesicle epithelium which does not regress markedly after castration in the puberal or adult hamster may be maintained to some extent by androgens from the adrenal cortex (Ortiz, 1953). Evidence from the present study indicates that the adrenal androgens, if they do exist, do not exert any influence on the epithelial lining of the seminal vesicle, since adrenalectomy coupled with castration

caused no greater degeneration of the seminal vesicle epithelium than did castration alone.

It becomes evident from a review of these results that adrenal androgens are not necessary for the persistence of a certain level of sexual activity in the castrated male hamster and it is probable that the same situation exists in other animals as well. It is conceivable that androgens may be secreted by normal tissues other than the gonads and adrenal cortex, but the evidence for this is slight. Thus Jacot and Selye (1952) demonstrated that ACTH given to adrenalectomized spayed rats stimulated the preputial glands which are also known to be sensitive to androgens and estrogens. However it was not determined whether the ACTH was acting directly on the glands or whether stimulation occurred indirectly by the activation of a tissue capable of elaborating androgen-like or estrogen-like substances. Until it is shown that such androgen secreting tissues actually exist, the frequent suggestion that postcastrational sexual behavior is due to extra-gonadal androgens should be viewed with considerable circumspection.

SUMMARY AND CONCLUSIONS

The present experiment was designed to investigate the role of the adrenal cortex in sexual behavior; in particular to determine its influence on the persistence of sexual activity in the castrate. It is concluded that:

1. DCA administered daily over a two month period in doses used to maintain adrenalectomized hamsters and up to 3 times this amount, had no andromimetic effect on sex behavior or on the seminal vesicles of the castrate.

2. Adrenalectomy in the mature male hamster has no effect on reproductive behavior when the animals are maintained in good health on DCA. The testes and seminal vesicles remained normal and successful impregnations occurred.

3. The sexual behavior that persists after castration is not dependent on extra-gonadal androgens from the adrenal cortex, for castrated-adrenalectomized males maintained on DCA behaved similarly to their castrated controls. The seminal vesicles were indistinguishable from those of the simple castrate.

REFERENCES

BEACH, F. A.: *J. Clin. Endocrinol.* **4**: 126. 1944.

BEACH, F. A.: Hormones and Behavior, New York, Paul B. Hoeber. p. 25. 1948.

BEACH, F. A. AND R. S. PAUKER: *Endocrinology* **45**: 211. 1949.

BOTELLA LLUSIA, J.: *Gynaecologia* **133**: 79. 1952.

DEMPSEY, E. W.: Handbook of Experimental Psychology, New York, John Wiley and Sons. p. 216. 1951.

FITZHUGH, O. G.: *Amer. J. Physiol.* **118**: 677. 1937.

FREED, S. C., B. BROWNFIELD AND H. M. EVANS: *Proc. Soc. Exper. Biol. and Med.* **29**: 1. 1931.

GAUNT, R.: *Amer. J. Physiol.* **103**: 494. 1933.
GREENE, R. R. AND M. W. BURRILL: *Proc. Soc. Exper. Biol. and Med.* **43**: 382. 1940.
HOOKER, C. W. AND V. J. COLLINS: *Endocrinology* **26**: 269. 1940.
HOWARD, E.: *Endocrinology* **38**: 156. 1946.
JACOT, B. AND H. SELYE: *Endocrinology* **50**: 254. 1952.
JAFFE, H. L.: *J. Exper. Med.* **38**: 107. 1923.
KINSEY, A. C., W. B. POMEROY, C. E. MARTIN AND D. H. GEBHARD: Sexual Behavior in the Human Female, Philadelphia, W. B. Saunders. p. 755. 1953.
MOORE, C. R.: *J. Clin. Endocrinol. and Metab.* **13**: 330. 1953.
MORGAN, C. T. AND E. STELLAR: Physiological Psychology, 2nd ed., New York, McGraw-Hill Book Co. p. 426. 1950.
ORTIZ, E.: *Anat. Rec.* **117**: 65. 1953.
PAUKER, R. S.: *J. Comp. and Physiol. Psychol.* **4**: 252. 1948.
PAUKER-WARREN, R. AND L. R. ARONSON: *Anat. Rec.* 113 no. 4 abst. 50. 1952.
ROBSON, J. M.: *J. Physiol.* **113**: 537, 1951.
SCHWARTZ, M. AND F. A. BEACH: *Amer. Psychol.* **9**: 466. 1954.
SELYE, H. AND S. ALBERT: *J. Pharm. and Exp. Therap.* **76**: 137. 1942.
SNYDER, J. G. AND L. C. WYMAN: *Amer. J. Phyisol.* **167**: 328. 1951.
SOLLENBERGER, R. T. AND J. B. HAMILTON: *J. Comp. Psychol.* **28**: 81. 1939.
STONE, C. P.: *J. Comp. Psychol.* **7**: 369. 1927.
STONE, C. P.: *J. Genetic. Psychol.* **40**: 296. 1932.

ALLOXAN DIABETES IN THE HAMSTER: DOSAGE AND BLOOD SUGAR CURVES[1]

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INTRODUCTION

DESPITE the extensive literature, only two articles, Harris, Anderson and Chen (1946) and Phatak, Lal and Everett (1950) deal with alloxan and the hamster. In neither was the method of injection, the exact diabetic dose, the form of the blood sugar curve, the duration of the diabetic symptoms or the survival time made clear. Since all of this information was pertinent to proposed studies employing the hamster cheek pouch in diabetic animals, the work reported here was undertaken.

MATERIALS AND METHODS

One hundred male and an equal number of female golden hamsters of 40 to 130 gm initial weight bred in this laboratory and maintained on Purina Laboratory Chow were used. Eastman alloxan (30 to 200 mg./kg.) was injected into the orbital venous sinus (Tassoni, 1955). Blood was drawn from the same site 2, 4, 6, 8, 12, 24, 48, and 72 hours after injection. Glucose was determined by the Reinecke (1942)method.

OBSERVATIONS

Alloxan Dosage. The results of these studies are adequately summarized in Table 1.

TABLE 1. RESPONSE OF HAMSTERS TO INTRAVENOUS ALLOXAN

Fasted 48 hrs.	Ineffective	30–40 mg./kg.
	Diabetogenic	45–50 mg./kg.
	Lethal	55–90 mg./kg.
Non-fasted	Ineffective	10–50 mg./kg.
	Diabetogenic	60–70 mg./kg.
	Lethal	80–100 mg./kg.

Blood Sugar Levels. Figure 1 represents the mean values for 140 determinations on 39 diabetic hamsters, both fasted and fed before treatment. While there is considerable variation in the magnitude of the rise in blood sugar (Table 2) and in the time required to reach a maximum, it was ob-

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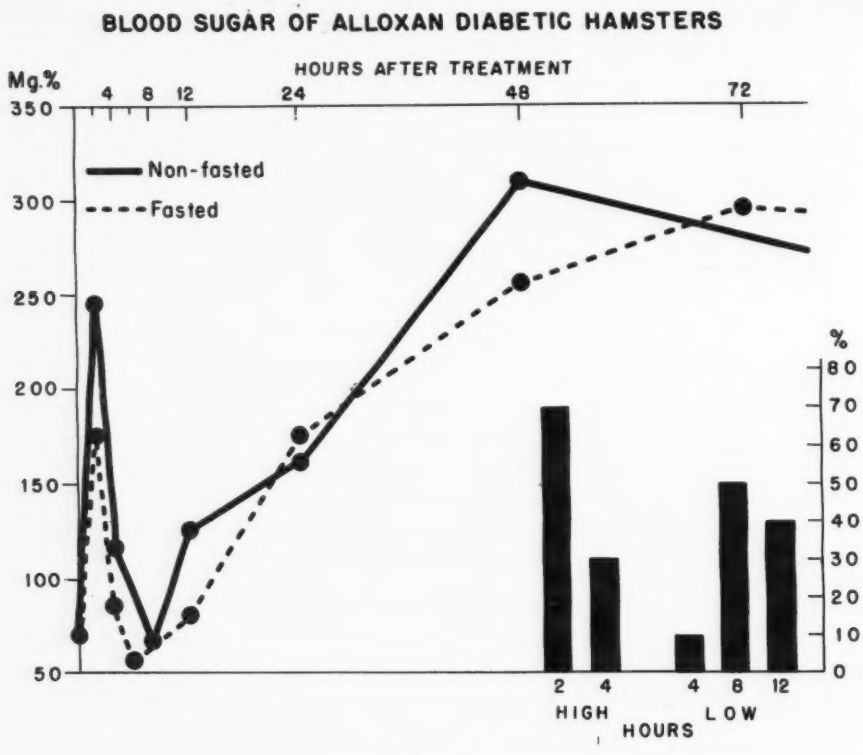

FIG. 1

served that more than 70% reached a peak in two hours (Fig. 1) after injection. The remaining animals attained their summit within 4 hours. After the primary rise, glucose levels decline. Nearly half of the animals

TABLE 2. RANGE OF BLOOD SUGAR VALUES FOR DIABETOGENIC AND LETHAL DOSES OF ALLOXAN OVER A 72 HOUR PERIOD

Time—in hours after injection; L—Low; M—Mean; H—High

Time	Normal			Diabetogenic						Lethal					
				Non-Fasted			Fasted			Non-Fasted			Fasted		
	L	M	H	L	M	H	L	M	H	L	M	H	L	M	H
	60	70	90												
1										60	120	300	60	145	210
2				80	245	370	100	175	250	70	210	430	90	225	380
4				50	115	240	70	85	120	50	130	460	90	210	370
8				50	65	110				50	100	480	50	95	210
12				50	125	270	50	80	240	50	55	70	50	105	280
24				50	160	380	50	175	500	50	170	500	50	215	470
48				90	310	500	130	255	470	50	245	500	50	180	500
72							240	295	370	50	205	500	60	330	500

fall to their low at the time indicated, although a few may reach it as early as 4 hours and between 30 and 40% may delay this phase for as long as 12 hours. The low is followed by an increase which continues through the second day. Beyond this time the non-fasted tend to decline slowly through the fourth day while the fasted rise for another 24 hours before sloping off.

Effects of Lethal Doses. The general configuration of the blood sugar curves for diabetic and lethally-injected animals is quite similar, the chief differences lying in the fact that the hypoglycemic phase tends to appear 2 to 4 hours later in the moribund and at the same time, particularly after the first day, the curve is more erratic with a wider range of values in this group (Table 2).

Survival Time. Approximately 50% of the animals died in the course of the third day after receiving a lethal dose. Fatalities began on the second day when 10% succumbed. Thirty-five percent died during the fourth day while 5% survived into the fifth day.

DISCUSSION

Although Harris, Anderson and Chen (1946) used the intravenous approach in the hamster and Sullivan and Everett (1952) described an important advance, it was not until the development and subsequent modification of the ocular sinus technique (Tassoni, 1955) which facilitates intravenous injections and periodic sampling of blood from the same animal, that a complete study of the effects of this drug on the hamster became possible. It is interesting to note that, in spite of technical difficulties, the aforementioned group had estimated the ideal dose to be 60 mg./kg. However, these investigators did not show how critically narrow the dose range is nor did they indicate a difference in the diabetic dose for fasted animals.

The single intravenous dose range which we found effective falls within the limits reported for other small rodents.

Our normal blood sugar values appear considerably lower than those usually given for the hamster. However, it should be remembered that all of our samples were taken under anesthesia; that our source was a subdural sinus rather than superficial veins; that, for the most part, other investigators drew blood from conscious, struggling animals under stress and excitement which would tend to increase the level of glucose.

Although Goldner and Gomori (1943, 1944), Hard and Carr (1944) and Weinglass *et al.* (1945) mention the use of fasted animals, it was Kass and Waisbren (1945) who first stated that fasting rats 48 to 60 hours before injection will render them almost 100% susceptible to alloxan. In attempting to ascertain whether this might also hold true for the hamster, we discovered that our normal diabetic dose was lethal for fasted animals. Since Lazarow, *et al.* (1946, 1948, 1949) and others have indicated the influence of cysteine, glutathione, nicotinic acic and sodium upon the deleterious effects of alloxan, it seems likely that deficiencies of certain of these in

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starved animals account for the lower dose requirements in the latter.

The triphasic curve shown here so closely conforms to that seen in other animals, further comment is unnecessary. However, it should be emphasized that none of our hamsters showed extremely low blood sugar or gave any clinical signs of hypoglycemia. The fact that both peak and low points for fasted animals lie below the counterparts for fed animals perhaps needs explanation. Assuming that fasting hamsters possess less glycogen reserves, the initial glycogenolysis caused by the injection would mobilize less, thus producing a smaller peak than is the case of the fed animal. Conversely, equal, or nearly equal amounts of insulin produced by and released from the islets of both groups under the influence of the drug would tend to produce a lower hypoglycemic phase, earlier, in the fasteds, since the latter contained less glucose.

Although some reports have shown that lethal doses kill within a few hours, the hamster is similar, within the range used, to those animals having a 3 to 6 day survival period.

Our observations show that diabetic and lethal doses produce comparable changes in blood sugar, particularly within the first 24 to 36 hours. Experimentation among other animals has indicated that the curve is changed neither in form nor amplitude by increasing the dose above the effective level.

SUMMARY

In hamsters fasted 48 hours, 45–50 mg./kg. of alloxan, given by orbital sinus, produces persistent hyperglycemia. For fed animals, 60–70 mg./kg. is required. Blood sugar levels reached 175 mg.% for the former and 245 mg.% for the latter in 2–4 hours, fell to 55 and 65 mg.% respectively in 6–8 hours, rose again to 255 and 310 mg.% at 48 hours and remained for both groups between 250 and 300 mg.% for the next two days. Lethal doses produce similar changes during the first 24–36 hours, with a survival period of 2 to 5 days.

REFERENCES

GOLDNER, M. G. AND G. GOMORI: *Endocrinology* **33**: 297. 1943.

GOLDNER, M. G. AND G. GOMORI: *Proc. Soc. Exper. Biol. and Med.* **55**: 73. 1944.

HARD, W. L. AND C. J. CARR: *Proc. Soc. Exper. Biol. and Med.* **55**: 214. 1944.

HARRIS, P. N., R. C. ANDERSON AND K. K. CHEN: *J. Pharmacol. and Exper. Therap.* **87**: 382. 1946.

KASS, E. H. AND B. A. WAISBREN: *Proc. Soc. Exper. Biol. and Med.* **60**: 303. 1945.

LAZAROW, A.: *Proc. Soc. Exper. Biol. and Med.* **61**: 441. 1946.

LAZAROW, A.: *Proc. Am. Diabetes A.* **9**: 409. 1949.

LAZAROW, A., J. W. PATTERSON AND G. F. LEVEY: *Science* **108**: 308. 1948.

PHATAK, N. M., D. LAL AND F. G. EVERETT: *J. Pharmacol. and Exper. Therap.* **98**: 26. 1950.

REINECKE, R. M.: *J. Biol. Chem.* **143**: 351. 1942.

SULLIVAN, R. E. AND F. G. EVERETT: *J. Dent. Res.* **31**: 151. 1952.

TASSONI, J. P.: *Anat. Rec.* **121**: 453 (Abst.) 1955.

WEINGLASS, A. R., E. G. FRAME AND R. H. WILLIAMS: *Proc. Soc. Exper. Biol. and Med.* **58**: 216. 1945.

CORTICOSTEROID SECRETION IN THE ADRENAL VEIN OF THE NON-STRESSED DOG EXPOSED TO COLD

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THE sensitivity of the adrenalectomized animal to exposure to cold has been well documented (Hartman and Brownell, 1949), and has also been used as an assay for cortical hormones (Selye and Schenker 1938). Indirect measurements of adrenocortical function, such as adrenal size (Hartman, Brownell, and Crosby, 1931) and fall in ascorbic acid and cholesterol content of the adrenal gland (Long, 1947) have indicated adrenocortical stimulation on exposure to cold.

The development of procedures for measuring cortical steroids in blood (Nelson and Samuels, 1952) and for obtaining adrenal venous blood samples from the non-stressed dog (Hume and Nelson, 1954) have made possible the present study of adrenocortical steroid secretion in the adrenal venous blood of the non-stressed dog exposed to cold. Corticosteroid secretion has been determined in the dog while surgically stressed, during convalescence, while exposed to $-10°$ C, and when given ACTH while at room temperature or during exposure to the cold environment.

METHODS

Sixteen male mongrel dogs weighing between 9 and 24 kilograms were subjected to adrenal cannulation under nembutal or ether anesthesia by a procedure which has previously been described (Hume and Nelson, 1955). Adrenal venous blood was collected for periods of exactly one or two minutes intermittently during the period of operative trauma, and after periods of a few hours to 10 days dollowing operation. While samples were not being collected, the cannula was filled with 4% heparin in physiological saline solution. During the collection of samples, except when anesthetized for the operation, the animals were only gently restrained. In most cases this did not appear to be a very stressful procedure although the animals did apparently have some sensation when the "choker" on the adrenal vein medial to the gland was tightened. After adrenal secretion had fallen to a basal level (usually the first or second post-operative day), the animals were placed in a cold room at $-10°$ C for periods varying from 1 to 33 hours. The ability of the adrenal to respond to ACTH was tested either while the animal was in the cold room or immediately after return to a warm environment. ACTH was administered intravenously in a single 40 unit dose.

Corticosteroids in the adrenal effluent were determined by the method of Nelson and Samuels (1952) for 17-hydroxycorticosteroids in blood. In all cases whole blood rather

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than plasma was used for the determinations. The samples were refrigerated at 4° C following collection and the analyses carried out within 1–3 days.

RESULTS

The results of determination of 17-hydroxycorticosteroid secretion under various conditions are presented in Table 1. The high levels of steroid secretion on the day of operation are apparent. The secretion on the day following operation appeared to be elevated in some individual dogs, but this was not statistically significant for the group. There was a significant difference between the values obtained when operation was performed under ether rather than nembutal anesthesia (P value $<.001$). Exposure to $-10°$ C caused no consistent rise in corticosteroid secretion, but exposure to room temperature following cold appeared to produce a steroid release. In every case the intravenous administration of ACTH produced a considerable increase in steroid secretion.

TABLE 1. EFFECT OF COLD, ABDOMINAL LAPAROTOMY, ANESTHESIA, AND ACTH ON CORTICOSTEROID SECRETION AS MEASURED BY MINUTE OUTPUT IN ADRENAL VEIN OF 16 DOGS

	No. of Determinations	17-Hydroxycorticosteroids secreted/min. (μg. $\pm$S.E.)	Significance of difference from mean secretion (P)
Day of operation (ether anesthesia)	53	10.3 ± 0.7	$<.001$
Day of operation (nembutal anesthesia)	37	6.1 ± 0.8	$<.01$
Day after operation	24	3.1 ± 0.8	$>.8$
Mean secretion (no procedure)	99	2.8 ± 0.6	—
During exposure to $-10°$ C cold	75	2.1 ± 0.4	$>.3$
Warming following cold exposure	28	7.1 ± 1.1	$<.01$
During first hour following ACTH administration	32	15.2 ± 1.3	$<.001$

In compiling the data for Table 1, it was difficult to express the time relationships which in a study such as this are extremely important Therefore, protocols of experiments on two of the sixteen dogs studied are presented in Figures 1 and 2.

Dog A (Fig. 1) on the day of operation had a typically high level of corticoid secretion which fell to a basal level on the second day. During a 7-hour exposure to $-10°$ C he had bursts of steroid secretion which were out of the expected normal range, but on the fourth day the level was also elevated for a short period of time while the animal was at room temperature. Marked increases in secretion on the fourth, seventh, and ninth days following intravenous ACTH administration demonstrated the continuing ability of adrenal to respond to stimulation. Exposure to $-10°$ C for a period of 7 hours on the ninth day caused no increase in corticoid secretion.

Dog B (Fig. 2) had three elevated values during exposure to $-10°$ C on the fifth and sixth days, but six values during the same period were not elevated nor was a value on the third day after six hours in the cold. A

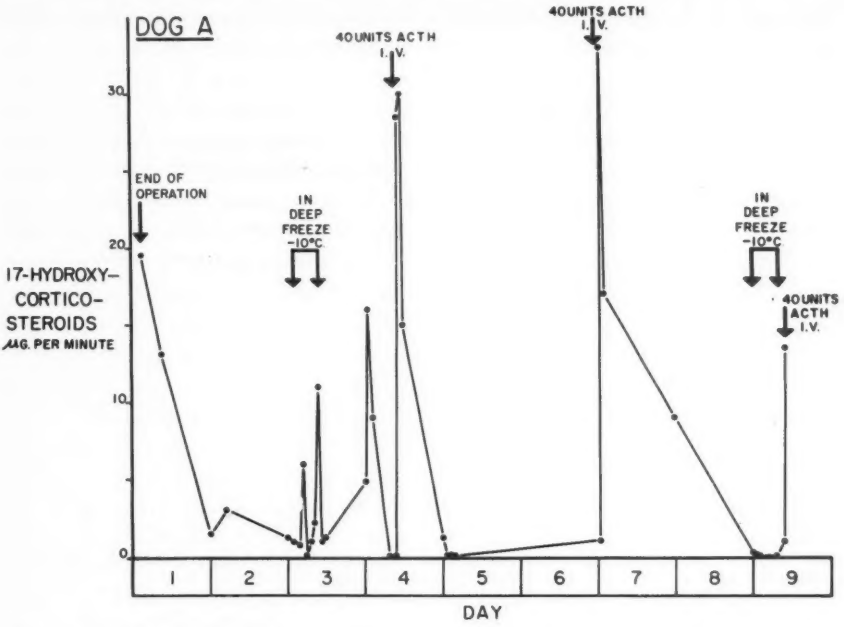

FIG. 1. Minute secretion of 17-hydroxycorticosteroids in the adrenal vein of a dog exposed to trauma, −10° C, and given 40 units ACTH intravenously in single doses.

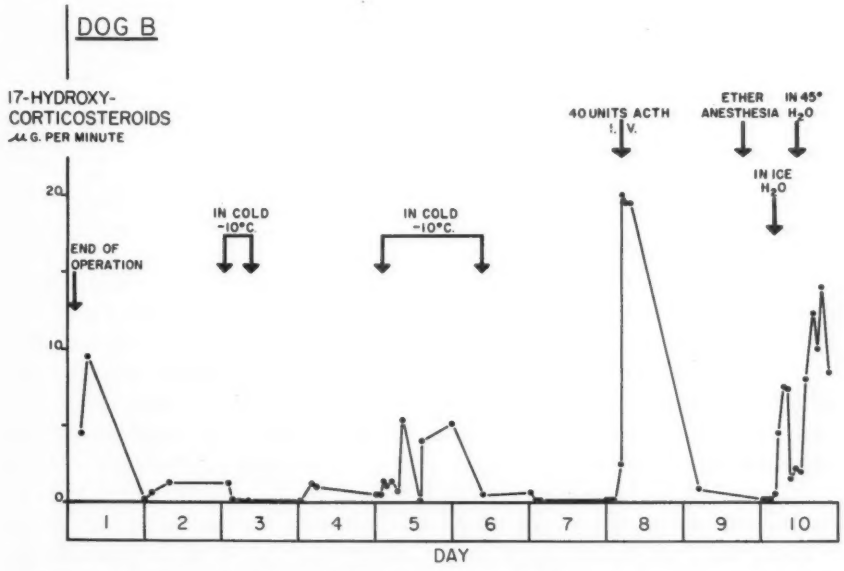

FIG. 2. Secretion of 17-hydroxycorticosteroids per minute following exposure to trauma, cold, ACTH, ether anesthesia, cold under anesthesia to the point of hypothermia, and warming following cold.

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marked steroid secretory response to immersion in ice water under ether anesthesia and upon rewarming following hypothermia was seen on the ninth day.

The effect of continuing exposure to −10° C for periods up to 33 hours is illustrated in Figure 3. No progressive increase in steroid output was seen. On a number of occasions the animals were found to have short bursts of adrenocortical secretion which did not seem to be related to any specific procedure. These periods of secretion were, however, generally

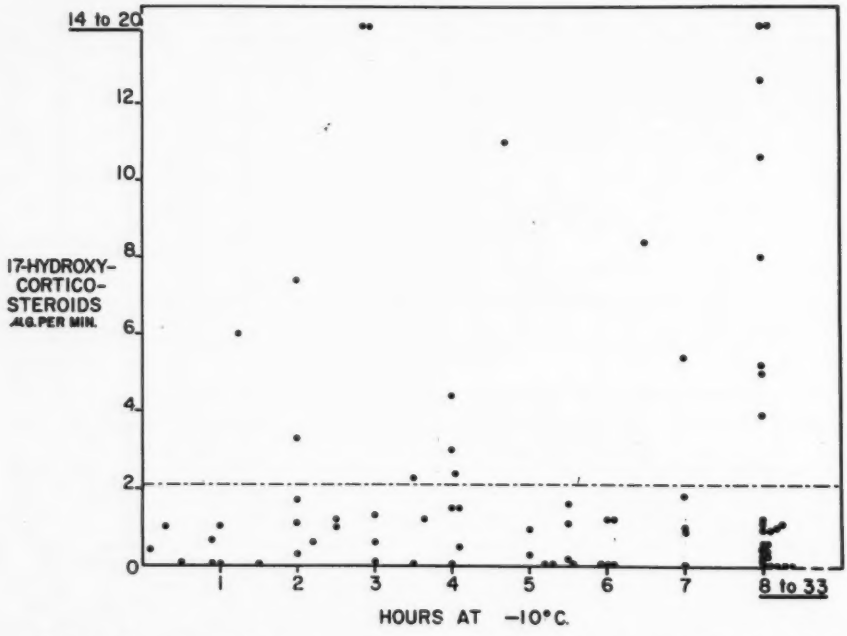

FIG. 3. Micrograms of 17-hydroxycorticosteroids secreted per minute in adrenal vein of dog following exposure to −10° C for varying lengths of time. Dotted line represents mean secretion during this period.

brief and occurred both at room temperature and during cold exposure.

On two occasions ACTH was administered to dogs which were in the −10° C environment and typical increases in corticosteroid secretion resulted. Temperature measurements taken during the cold exposure demonstrated the ability of the animals to maintain normal body temperature under these conditions.

DISCUSSION

The relatively high rate of corticosteroid secretion of the surgically stressed dog has been well documented (Vogt, 1943, and Nelson, Reich and Samuels, 1951). The technique used in the present study, which allows

estimation of corticoid secretion per minute, has demonstrated the relatively low output in the resting state (Hume and Nelson, 1954). No direct measurements of corticosteroid output in response to cold have been reported, but Thorn and Laidlaw (1953) failed to demonstrate increases in urinary 17-hydroxycorticosteroids when normal human subjects were exposed to 4° C for a period of four hours.

Corticosteroid secretion is undoubtedly influenced by many factors. The lability of the system is demonstrated by the many short bursts of secretory activity seen in the present study during periods when no specific stress was applied. The relatively short period of increased secretion under these circumstances is similar to that seen when ACTH in doses of 2–5 milliunits is administered to hypophysectomized dogs (Nelson and Hume, 1955). It has been demonstrated that ACTH given in these small doses will produce increased corticoid secretion for periods of only 15–20 minutes. It would thus appear that the pituitary glands of these animals were able to release small amounts of ACTH in response to minimal stimuli. During periods of prolonged stress, such as surgical laparotomy, rather large quantities of ACTH appeared to be secreted over fairly long periods of time (Nelson and Hume, 1954).

Although exposure to cold environment is reported to be a definite stress to the adrenalectomized rat (Selye and Schenker, 1938), the dogs in this study did not consistently secrete increased quantities of 17-hydroxycorticosteroids during exposure to –10° C. It is worth noting that the rat is reported to be more sensitive to cold stress than is the dog. The surface-volume ratio and relative amount of insulation (fur and subcutaneous fat) of the dog compared with the rat makes the former better able to withstand cold without an increased metabolic rate (Robinson, 1952). Although in a few animals in this study there appeared to be more frequent bursts of corticoid secretion at −10° C than at room temperature, this was not consistent and the average secretion during periods of cold exposure was not significantly elevated over the basal level. The possibility that compounds other than 17-hydroxycorticosteroids were secreted in response to the cold stress cannot be absolutely ruled out but this seems unlikely. The animals always responded well following ACTH administration and the chief corticosteroid secreted by the dog is known to be 17-hydroxycorticosterone which is measured by the techniques used.

Hypothermia has been shown to decrease adrenal response to ACTH (Egdahl, Nelson and Hume, 1955) but the animals in this study were not hypothermic when exposed to −10° C and they always responded to ACTH stimulation while in the cold. Removal to room temperature following cold exposure appeared to be a stimulus to the pituitary-adrenal system, and an increase in corticoid output was seen during these periods.

If neither type of steroid secreted, nor the effect of cold on adrenal responsiveness (hypothermia) can explain the apparent differences between

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the data described here and those of other workers, other explanations must be investigated. The basal secretion of the adrenal gland under these conditions may be sufficient to maintain the animal and no increase in corticosteroid secretion may be necessary to adjust adequately to the cold environment although complete deprivation might lead to fall in body temperature and death (Hartman, Brownell, Crosby, 1931). On the basis of the values obtained for the secretion from one adrenal gland for a period of one minute, one can calculate that both glands would secrete about 6 mg. of 17-hydroxycorticosterone per 24 hours. This is a substantial amount of hormone and might be expected to offer considerable protection against the stress of the cold exposure. Under conditions of more severe stress or tissue damage (burns, surgery, etc.) a greater demand for hormone is present and increased secretion of ACTH and adrenocortical steroids occurs.

SUMMARY

17-Hydroxycorticosteroid secretion per minute in the adrenal vein has been determined in 16 dogs during the surgical procedure of placing a permanent cannula in the lumbo-adrenal vein, through convalescence, and for periods of a few hours to 10 days following operation. These dogs were exposed to $-10°$ C for periods of 1–33 hours from the third to the ninth post-operative day and no significant increase in steroid output above the basal level was seen. Definitely elevated values were found during surgery (higher with ether than nembutal anesthesia), following warming after cold exposure, and after ACTH administration. Investigations by other workers have demonstrated the sensitivity of the adrenalectomized rat to cold exposure. The failure of the dogs in this study to secrete increased corticoids under these conditions may mean that the basal secretion of approximately 6 mg. per 24 hours is sufficient to prevent pituitary stimulation and that the dog is less sensitive to exposure to $-10°$ C than is the rat.

REFERENCES

EGDAHL, R. H., D. H. NELSON AND D. M. HUME: *Science* **121**: 506. 1955.

HARTMAN, F. A. AND K. A. BROWNELL: *The Adrenal Gland.* Lea and Febiger, Philadelphia, 1949.

HARTMAN, F. A., K. A. BROWNELL AND A. A. CROSBY: *Am. J. Physiol.* **98**: 674. 1931.

HUME, D. M. AND D. H. NELSON: *Fed. Proc.* **13**: 73. 1954.

HUME, D. M. AND D. H. NELSON: in Surgical Forum—Clinical Congress of the American College of Surgeons, 1954. Vol. 5, W. B. Saunders Co., p. 568, Philadelphia, 1955.

LONG, C. N. H.: in Recent Progress in Hormone Research, Vol. 1, p. 99, 1947.

NELSON, D. H. AND D. M. HUME: *J. Clin. Endocrinol. and Metab.* **14**: 781. 1954.

NELSON, D. H. AND D. M. HUME: *Endocrinology* **57**: 184. 1955.

NELSON, D. H. AND L. T. SAMUELS: *J. Clin. Endocrinol. and Metab.* **12**: 519. 1952.

ROBINSON, S.: *Ann. Rev. Physiol.* **14**: 73. 1952.

SEYLE, H. AND SCHENKER: *Proc. Soc. Exper. Biol. and Med.* **39**: 518. 1938.

THORN, G. W. AND J. C. LAIDLAW: *Trans. Am. Clin. and Climatological Assoc.* **179**: 199. 1953.

THE EFFECT OF CASTRATION ON THE WEIGHT AND COMPOSITION OF THE MUSCLES OF THE GUINEA PIG[1,2,3]

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ONE of the expressions of the protein anabolic effect of the androgens is an increase in muscle mass. This is strikingly demonstrated by the temporal muscle of the guinea pig (Papanicolaou and Falk 1938; Kochakian, Humm and Bartlett 1948; Scow and Roe 1953). It seemed that possibly the temporal muscle is not representative of all of the muscles of the body. Therefore, a detailed study has been made of the effect of castration on the rate of growth of 49 different muscles of the guinea pig.

PROCEDURE

Male guinea pigs of the inbred Hartley strain (Tumblebrook Farms, Brant Lake, New York) were castrated at 534 (457–573) gm. body weight. A group of normal animals were killed at the time of castration and paired groups of normal and castrated pigs at intervals of 6, 12 and 27 weeks later. In addition, a group of normal guinea pigs was castrated 21 weeks later than the first group and killed at the same time as the 27-week groups. There were 8 animals in each group. The guinea pigs were maintained four in a cage in an air-conditioned room at 22–23° C and with constant artificial light 12 hours per day. They were fed Superior Rabbit Checkers ad libitum and 30–40 gm. of carrot and a 25 mg. tablet of Vitamin C twice per week. They were weighed three times per week and animals that did not show good growth curves were discarded.

At autopsy the internal organs were weighed and the remaining viscera and abdominal fat were removed. The carcass then was placed in a plastic bag and kept frozen until time of dissection. The organs and muscles were weighed on appropriate Roller-Smith torsion balances. The muscles were dried at 90° for 48 hours, weighed and dissolved in 5 to 25 ml. of 50% (w/v) aqueous potassium hydroxide and an equal volume of ethyl alcohol and made to 25, 50 or 100 ml. in accordance with the weight of the muscle. Nitrogen determinations were made on suitable (1–5 ml.) aliquots by the micro-Kjeldahl procedure.

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[2] Parts of these data were presented at the Endocrine Society meetings, June 3, 1955, at Atlantic City, New Jersey.

[3] The authors are greatly indebted to Dr. Alice Brues for her advice and suggestions concerning the anatomical aspects of this study and to Don Cockrell for technical assistance.

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RESULTS

Carcass and Internal Organs: Castration did not produce an immediate effect on the rate of growth (carcass weight) (Fig. 1) but after 6 weeks a definite decrease began to appear. Furthermore, the guinea pigs castrated when adults and autopsied at the 27-week period also showed a decrease in carcass weight. The total body weight showed a parallel effect.

The rates of growth of the liver, kidney, spleen and thymus (Fig. 1) were not altered by castration. After 27 weeks, however, the thymus of the

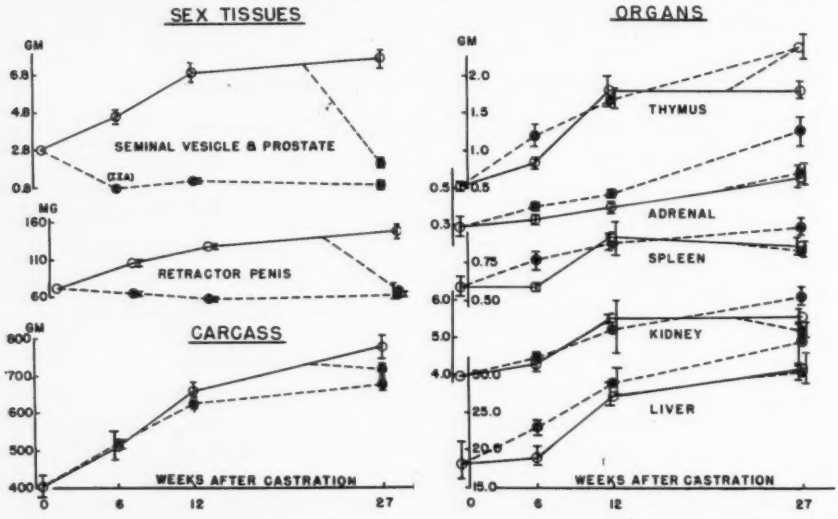

FIG. 1. Effect of castration on the carcass and organ weights of the guinea pig. Castration at 3–4 months of age and 534 (457–573) gm. body weight. The muscles from both the left and right sides were removed. One half the averages of both muscles except for the retractor penis and obliques with the standard errors of the mean are presented. The solid lines represented the values of the normal animals and the broken lines those of the castrated guinea pigs.

castrated animals was larger than normal in both the early and late castrated groups.

The adrenals of the castrated guinea pigs became progressively greater with duration of castration (Fig. 1).

The seminal vesicles and prostates (Fig. 1) showed the expected sharp decline in weight to a minimum value within 6 weeks. The weight of these organs at the time of castration indicated that sexual development had already begun.

Retractor Penis: This muscle ceased to grow immediately after castration (Fig. 1) but did not show the same degree of regression as the accessory sex organs. A search was made for the leavtor ani muscle because of its known marked response to castration (Wainman and Shipounoff 1941;

Eisenberg, Gordan and Elliott 1949) but it was found[3] to be only a vestigial muscle in the guinea pig.

Head and Neck: The digastric, masseter and temporal muscles (Fig. 2) responded to castration in a manner similar to the retractor penis except that the digastric and temporal muscles did not show a significant regression.

The neck muscles (clavotrapezius, levator scapulae and sternomastoid) continued to grow but at a slower rate for another 6 weeks after castration and then no further growth occurred.

Chest: The pectoral muscles and the serratus anterior (Fig. 2) showed a continued but a decreased rate of growth.

Shoulder and Back: These muscles (Fig. 2) showed a progressively decreasing rate of growth after castration.

Scapula: Castration produced a decreased rate of growth of these muscles (Fig. 2) but the difference between the normal and castrated animals was not significant until after 12 weeks of castration.

Thorax: The heart (Fig. 2) showed a decreasing rate of growth as a result of castration but the difference did not become significant until the 27-week period. The diaphragm (Fig. 2) showed a variable response.

Body wall and Spine: Castration decreased the rate of growth of these muscles (Fig. 2). The effect was sooner and greater in the psoas muscles.

Forearm: The differences between normal and castrated animals in the rate of growth of these muscles (Fig. 2) were small and did not manifest themselves until the 27-week period.

Hip: The gluteus muscles, semitendinosus and biceps femoris (Fig. 2) showed a decrease in rate of growth within 12 weeks after castration. The semimembranosus muscle was affected after 27 weeks and the caudofemoralis showed a questionable effect.

Thigh: The pectineus and adductor brevis muscles (Fig. 2) were not changed by castration. The other muscles showed varying degrees of decrease but only after 27 weeks of castration.

Shank: The flexor hallucis longus, tibialis anterior and the gastrocnemius muscles (Fig. 2) showed a decreased rate of growth after castration. The effect of castration on the other muscles did not become apparent until after 27 weeks. The differences in every instance were not very great.

Castration of adult animals: The removal of the testis 21 weeks later produced almost the same changes in 6 weeks as noted after 27 weeks of castration in the young animals except that the adrenals did not increase and the heart, sternomastoid, gracilis and semimembranosus muscles did not regress (Fig. 1, 2).

Moisture content of muscles: No remarkable change in moisture content of the various muscles was observed (Table 1), even in those muscles which were markedly affected in size as a result of castration. The changes in the

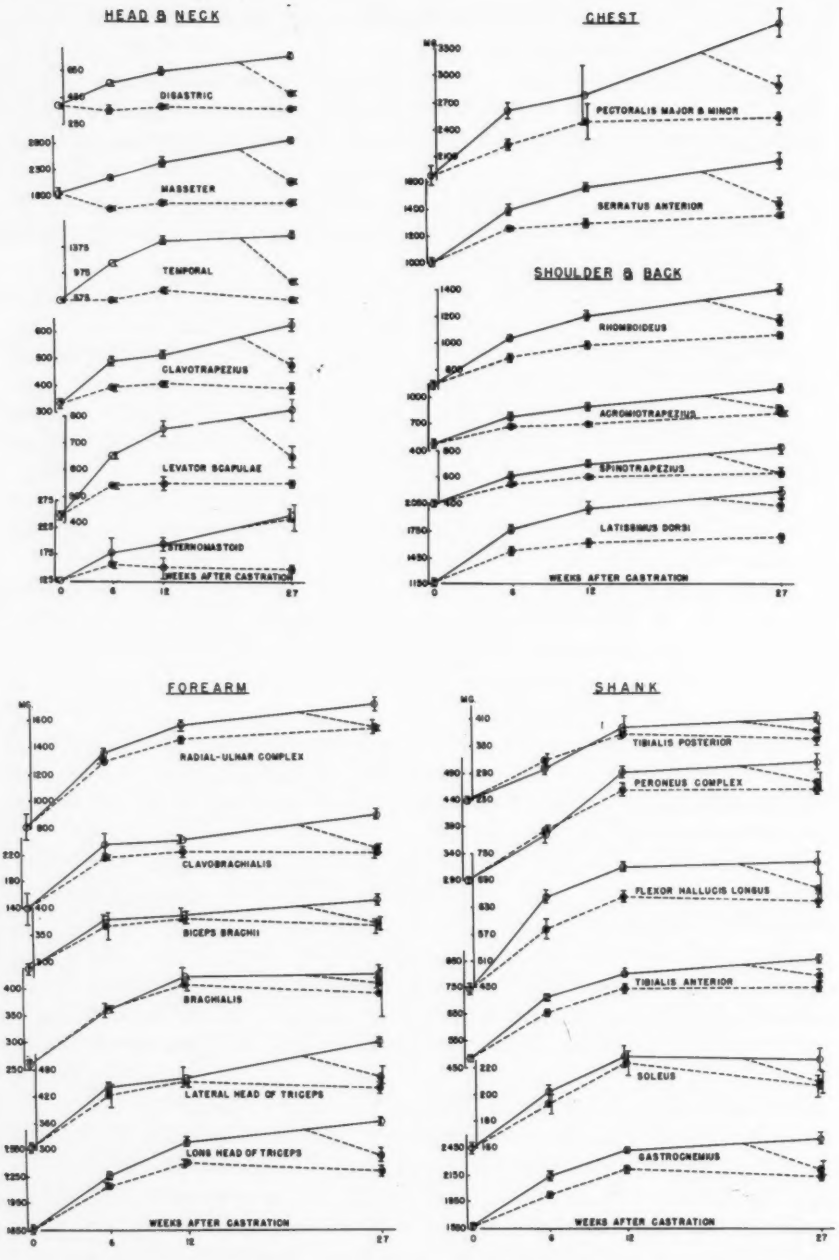

FIG. 2. Effect of castration on the muscles of the guinea pig. See Fig. 1 for details.

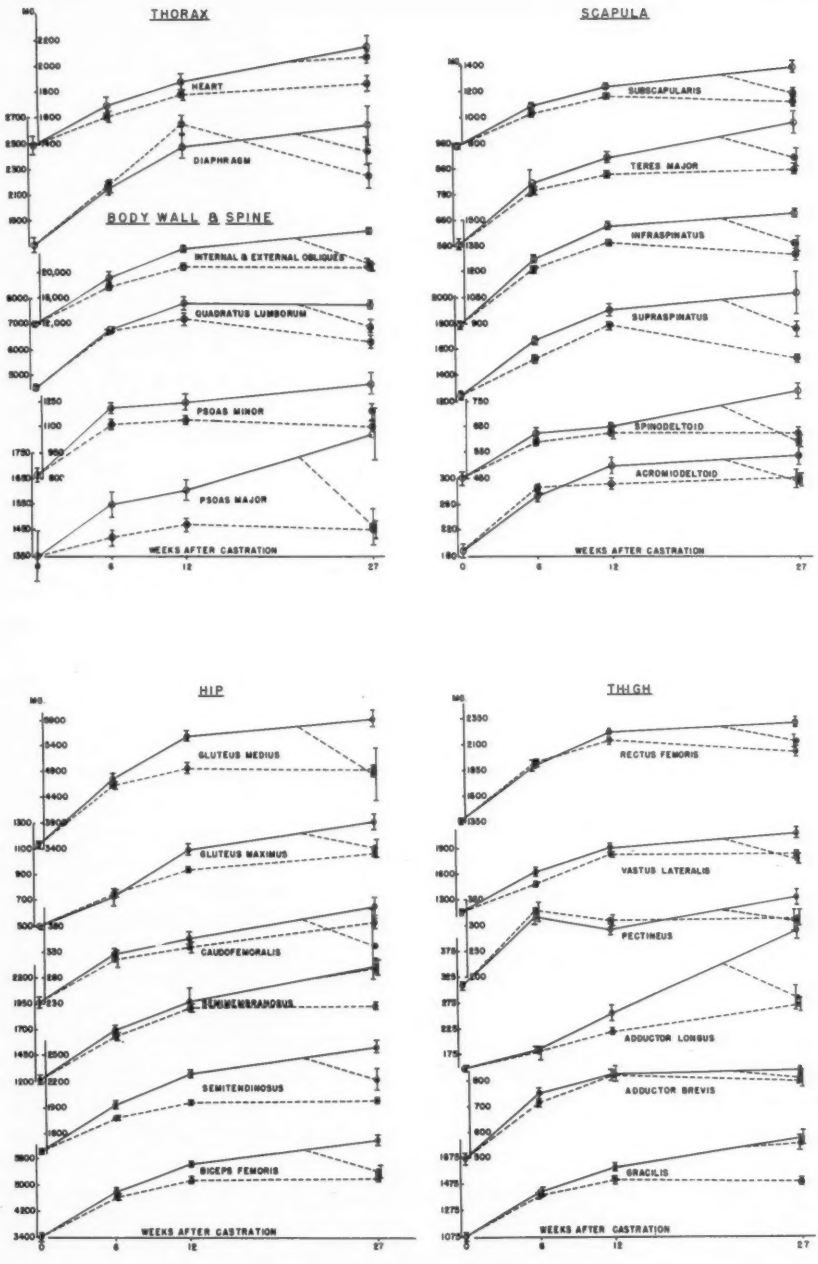

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TABLE 1. THE MOISTURE CONTENT OF GUINEA PIG MUSCLES

Muscle	Duration of castration							
	0 weeks	6 weeks		12 weeks		27 weeks	
	Normal %	Normal %	Castrate %	Normal %	Castrate %	Normal %	Castrate %
Retractor Penis				75.4	72.1	72.9	72.8
Head and Neck							
Digastric	76.0(72.0–78.4)	77.6	76.7	76.6	74.4	76.1	74.8
Masseter	75.4(72.9–76.7)	77.6	76.3	77.5	75.4	76.2	74.9
Temporal	75.0(73.6–76.7)	76.9	75.9	77.8	76.9	75.3	74.0
Clavotrapezius	75.5(72.8–78.4)	77.6	76.6	75.8	74.4	76.5	75.4
Levator scapulae	75.2(71.1–77.7)	76.8	76.5	75.7	73.2	73.8	75.0
Sternomastoid	76.8(72.7–79.0)	78.2	77.0	75.0	72.7	75.2	75.3
Chest							
Pectorals	73.7(71.3–76.6)	75.3	74.8	74.0	72.4	72.3	72.1
Serratus Anterior	75.7(74.0–77.9)	76.8	75.6	75.2	74.1	73.9	73.7
Shoulder and Back							
Rhomboideus	74.5(72.2–77.7)	76.2	75.0	74.2	72.7	74.2	72.7
Acromiotrapezius	71.7(69.2–73.2)	74.4	72.2	71.1	69.1	70.3	66.5
Spinotrapezius	71.4(69.1–74.3)	71.9	71.3	73.3	67.3	69.4	65.5
Latissimus dorsi	74.4(69.7–78.9)	75.2	74.4	75.4	72.7	72.4	70.0
Body Wall and Spine							
Diaphragm	75.8(73.6–78.6)	73.1	70.8	67.8	67.3	65.3	63.9
Internal and External Obliques	71.4(68.4–74.4)	74.8	73.7	72.1	73.0	70.7	70.6
Quadratus Lumborum	75.9(71.6–76.9)	76.2	75.6	75.4	75.1	74.6	73.7
Psoas minor	74.9(72.5–76.6)	75.6	74.9	73.3	73.4	72.7	71.9
Psoas major	75.2(73.5–76.5)	76.3	76.4	75.4	75.4	75.5	75.0
Scapula							
Subscapularis	73.6(68.8–76.8)	74.6	74.6	73.6	73.2	73.6	72.0
Teres major	73.8(69.0–76.9)	75.5	75.0	75.0	74.2	74.4	73.6
Infraspinatus	75.0(72.0–78.1)	75.9	75.7	75.5	74.5	74.9	73.9
Supraspinatus	76.3(74.7–77.8)	77.1	76.9	76.6	77.4	75.2	74.3
Spinedeltoid	73.0(69.8–74.0)	75.2	75.0	74.1	73.9	71.9	73.6
Acromiodeltoid	73.6(67.4–78.5)	74.9	75.0	72.1	70.0	71.9	73.1
Forearm							
Radial-ulnar Complex	68.3(64.9–73.2)	71.0	70.6	70.3	69.4	71.1	70.2
Clavobrachialis	74.4(71.5–76.7)	76.1	76.3	74.2	72.7	72.2	74.6
Biceps brachii	72.1(67.9–74.8)	74.3	74.2	72.9	73.2	73.4	73.9
Brachialis	73.0(69.3–76.6)	75.4	75.2	73.2	72.9	73.5	73.5
Lateral head of triceps	73.3(69.9–76.8)	75.3	75.1	74.4	73.3	74.2	73.3
Long head of triceps	75.5(72.7–77.7)	76.5	76.3	75.3	75.8	74.9	74.8
Hip							
Gluteus medius	76.7(75.4–78.6)	77.2	76.6	76.6	76.3	75.2	74.0
Gluteus maximus	70.0(65.9–72.0)	70.4	68.8	66.2	64.5	63.8	58.8
Caudofemoralis	77.0(74.3–78.7)	76.6	76.1	76.0	75.1	75.8	75.6
Semimembranosus	77.4(75.5–78.2)	77.3	76.5	77.0	76.7	75.6	75.4
Semitendinosus	76.0(73.9–77.2)	77.0	76.8	76.9	76.3	76.2	76.1
Biceps femoris	76.8(75.2–78.3)	77.3	76.5	76.3	76.1	75.6	74.8
Thigh							
Rectus femoris	76.4(74.5–77.5)	76.8	77.0	76.8	76.7	76.1	75.8
Vastus lateralis	75.6(75.2–76.9)	76.7	76.1	76.1	76.1	75.9	75.5
Pectineus	76.2(74.3–78.3)	77.3	76.8	76.3	76.0	76.1	76.1
Adductor longus	74.6(70.0–78.6)	75.8	74.6	75.0	74.6	73.6	73.0
Adductor brevis	75.6(74.2–77.3)	76.2	76.6	76.4	76.1	76.0	75.6
Gracilis	74.5(72.0–76.7)	75.0	74.6	74.5	74.4	74.6	74.3

TABLE 1 (*Continued*)

Muscle	Duration of castration							
	0 weeks	6 weeks		12 weeks		27 weeks	
	Normal %	Normal %	Castrate %	Normal %	Castrate %	Normal %	Castrate %
		Shank					
Tibialis posterior	73.7(72.5–76.5)	74.3	74.1	72.4	72.6	73.6	72.8
Peroneus	71.7(68.8–74.2)	73.3	72.9	72.7	72.3	73.1	72.4
Flexor hallicus longus	74.9(72.8–77.9)	74.6	74.8	75.4	75.4	75.0	74.5
Tibialis anterior	74.3(72.8–76.4)	76.2	75.6	75.4	74.7	75.3	75.8
Soleus	73.9(69.0–76.9)	72.9	75.0	73.3	71.9	72.8	71.6
Gastrocnemius	75.5(74.3–76.5)	76.5	76.1	76.1	76.0	75.5	75.8

shoulder and back region are suggestive but were not statistically significant.

The moisture content was not the same for every muscle but many muscles did have the same values. The progressively lower values, especially with age, in the diaphragm, gluteus maximus and probably in a few other muscles can be associated with the gross observation of greater fat content.

Nitrogen (protein) content of muscles: No remarkable change in concentration of nitrogen was observed (Table 2) even in those muscles which changed in size after castration. A few muscles, e.g., gluteus maximus showed a change in nitrogen concentration with age due apparently to a greater content of fat.

Many of the muscles possessed the same percentage of nitrogen but the values were not the same for all of the muscles.

Effect of castration and age on the "total" muscle mass: The total muscle mass (the sum of all the muscles studied except the heart and diaphragm) progressively increased in the normal guinea pigs but in the castrated animals no further increase occurred after the 12-week period (Table 3). Age produced the well-known decrease in moisture content in both groups of animals. The total muscle mass became progressively a smaller percentage of the body and carcass weights with age and that of the castrate group was less than that of the normal group.

Comparison of the size of the bones in normal and castrated guinea pigs: The residual soft tissue was easily removed from the carcass after autoclaving at 20 pounds for 10 minutes. Castration for 27 weeks had no influence on the size of the various members of the skeleton (Table 4, Fig. 3). Indeed, the bones of the two groups showed extremely close agreement. The depth of the temporal fossa, however, was noted to be greater in the normal than in the castrated guinea pigs. This effect, however, proved to be due to the regression after castration of the circumferential periosteum rather than an alteration in the skull. The difference was not apparent after autoclaving to remove all of the soft tissue.

TABLE 2. THE NITROGEN CONTENT OF GUINEA PIG MUSCLES

Muscles	Duration of castration							
	0 weeks	6 weeks		12 weeks		27 weeks	
	Normal %	Normal %	Castrate %	Normal %	Castrate %	Normal %	Castrate %
Retractor Penis		3.12	3.18	3.14	3.55	3.10	3.00
Head and Neck							
Digastric	3.05(2.82–3.24)	2.71	2.78	2.89	2.93	2.91	3.06
Masseter	3.20(2.98–3.35)	2.91	3.10	2.86	3.00	3.04	3.08
Temporal	3.15(2.97–3.32)	2.98	2.98	2.95	2.97	3.18	3.10
Clavotrapezius	3.09(2.91–3.20)	2.88	2.97	3.20	3.14	3.11	3.28
Levator scapulae	3.13(2.95–3.33)	2.93	3.01	3.16	3.09	3.19	3.23
Sternomastoid	3.00(2.82–3.25)	2.81	2.95	3.11	3.10	3.01	3.11
Chest							
Pectorals	3.41(3.06–3.45)	3.04	3.03	3.04	3.23	3.20	3.09
Serratus Anterior	3.06(2.72–3.31)	3.06	3.22	3.07	3.19	3.11	2.98
Shoulder and Back							
Rhomboideus	3.41(3.12–3.81)	3.05	3.15	3.19	3.24	3.08	2.95
Acromiotrapezius	3.56(3.30–3.80)	3.07	3.16	3.26	3.19	3.08	2.90
Spinotrapezius	3.35(3.14–3.45)	2.95	2.82	3.16	3.15	3.20	2.98
Latissimus dorsi	3.08(2.85–3.27)	3.01	3.15	3.11	3.14	3.15	3.27
Body Wall and Spine							
Diaphragm	2.87(2.37–3.34)	2.84	2.77	2.88	2.76	2.91	3.00
Internal and External Obliques	3.54(3.24–3.70)	3.09	3.15	3.00	3.02	3.00	2.99
Quadratus Lumborum	3.24(3.10–3.81)	3.03	3.09	2.95	3.06	3.03	3.09
Psoas minor	3.26(3.10–3.65)	3.19	3.31	3.11	3.22	3.17	3.18
Psoas major	3.25(2.87–3.73)	3.17	3.27	3.30	3.30	3.20	3.21
Scapula							
Subscapularis	3.08(2.84–3.31)	3.30	3.30	3.00	3.10	3.05	2.99
Teres major	3.22(2.79–3.65)	3.15	3.40	2.97	3.10	3.00	2.96
Infraspinatus	3.48(3.10–3.85)	3.17	3.22	3.22	3.29	3.27	3.00
Supraspinatus	3.13(2.83–3.46)	2.99	3.05	2.95	3.06	3.08	3.07
Spinodeltoid	3.40(2.96–3.75)	3.12	3.16	3.15	3.23	3.30	3.19
Acromiodeltoid	3.32(2.98–3.67)	3.16	3.15	3.28	3.29	3.24	3.06
Forearm							
Radial-ulnar complex	3.60(3.18–3.90)	3.69	3.72	3.42	3.65	3.38	3.19
Clavobrachialis	3.39(3.12–3.78)	2.93	3.06	2.72	3.24	3.22	3.18
Biceps brachii	3.38(3.02–3.75)	3.42	3.47	3.09	3.14	3.10	3.10
Brachialis	3.14(2.81–3.53)	3.17	3.23	2.96	3.05	3.00	2.89
Lateral head of triceps	3.18(3.03–3.46)	3.22	3.32	2.92	3.20	3.11	2.90
Long head of triceps	3.07(2.81–3.41)	3.22	3.19	2.92	3.06	3.01	2.97
Hip							
Gluteus medius	2.87(2.70–3.05)	2.87	3.00	2.86	2.83	2.84	2.72
Gluteus maximus	3.77(3.36–3.10)	3.32	3.27	2.71	2.73	2.81	2.60
Caudofemoralis	3.25(3.10–3.46)	3.05	3.19	3.11	3.22	3.13	3.04
Semimembranosus	2.86(2.76–3.06)	2.80	2.90	2.79	2.81	2.77	2.73
Semitendinosus	3.32(3.13–3.51)	2.87	2.93	3.08	3.11	3.08	3.04
Biceps femoris	2.91(2.76–3.08)	2.78	2.86	2.86	2.87	2.81	2.75
Thigh							
Rectus femoris	3.26(3.02–3.49)	3.13	3.18	3.08	3.17	3.13	3.13
Vastus lateralis	3.50(3.32–3.64)	3.14	3.27	3.12	3.17	3.12	3.06
Pectineus	3.32(2.98–3.51)	3.02	3.06	3.09	3.11	3.08	3.10
Adductor longus	3.23(2.94–3.46)	3.04	3.27	2.88	2.88	2.95	2.80
Adductor brevis	3.32(3.21–3.94)	3.03	3.12	3.09	3.11	3.05	2.96
Gracilis	3.36(3.08–3.60)	3.00	2.93	3.23	3.27	3.06	3.00

TABLE 2 (*Continued*)

Muscles	Duration of castration							
	0 weeks	6 weeks		12 weeks		27 weeks	
	Normal %	Normal %	Castrate %	Normal %	Castrate %	Normal %	Castrate %
		Shank					
Tibialis posterior	3.57(3.40–3.77)	3.38	3.39	3.36	3.26	3.26	3.25
Peroneus	3.80(3.57–4.06)	3.58	3.57	3.38	3.47	3.20	3.11
Flexor hallicus longus	3.48(3.34–3.66)	3.34	3.36	3.28	3.30	3.31	3.21
Tibialis anterior	3.49(3.27–3.66)	3.22	3.35	3.19	3.27	3.21	3.10
Soleus	3.38(3.09–3.63)	3.30	3.40	3.33	3.30	3.23	3.14
Gastrocnemius	3.31(3.25–3.36)	3.22	3.28	3.25	3.27	3.24	3.18

DISCUSSION

The removal of the testis has a variable effect on the various muscles of the body and can be categorized as follows: (1) Immediate cessation of growth with minor regression in some instances, (2) continued growth but at a slower rate and for variable periods after castration and (3) no significant effect or in proportion to change in body weight. It is of particular interest that specific muscles are influenced and to a varying degree and that some muscles are not influenced by the testis. The muscles that are most markedly affected are those in the head and neck region and retractor penis. The other muscles are in various parts of the body. It is difficult to associate these muscle changes with alterations in general activity, for only some of the leg muscles are affected and only to a small degree. Scow and Roe (1953) found no changes in the weight and myoglobin concentration of the hind leg muscles in castrated guinea pigs.

The effect of castration on the growth of specific muscles of the guinea pig apparently is a species phenomenon. The muscles of the rat respond to castration by a general change of all of the muscles in proportion to body weight (unpublished). On the basis of gross physical observations and some clinical observations on man after androgen administration, it might be inferred that the guinea pig corresponds rather closely to man with respect to the role of the testis in muscular development.

TABLE 3. EFFECT OF CASTRATION AND AGE ON THE "TOTAL" MUSCLE MASS OF THE GUINEA PIG

Duration of castration weeks	Total weight		H_2O		Per cent of			
					Carcass weight		Body weight	
	Normal, gm.	Castrate, gm.	Normal %	Castrate %	Normal %	Castrate %	Normal %	Castrate %
6	139	128	75.6	75.1	27.1	24.7	20.0	18.8
12	160	143	74.4	75.1	24.2	23.9	17.7	17.2
27	177	140	73.7	73.0	22.8	20.8	16.9	15.0

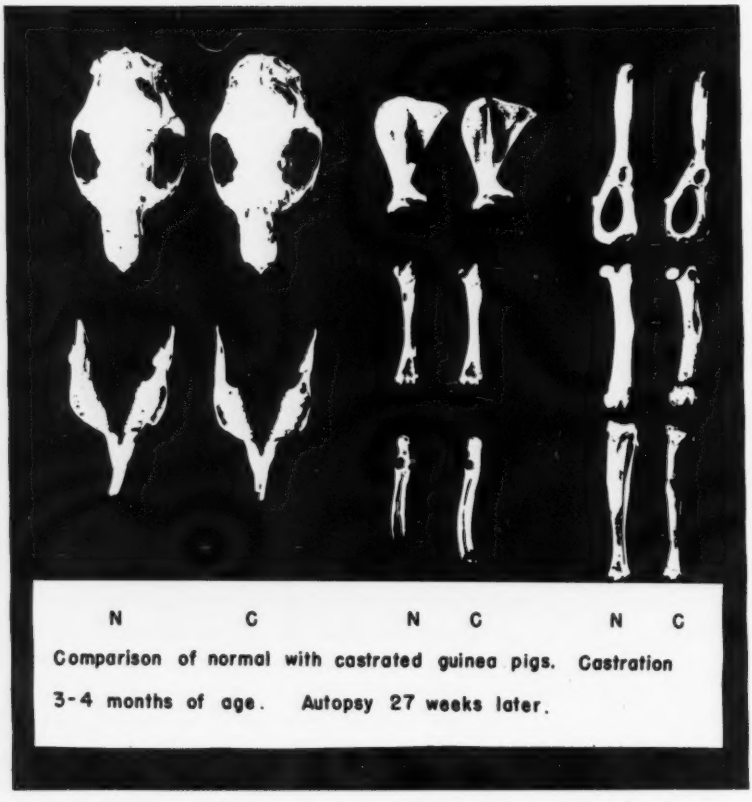

FIG. 3. The bones are from two paired normal (N) and castrated (C) guinea pigs. The bones and also the teeth showed no alteration as a result of castration (cf. Table 4).

TABLE 4. COMPARISON OF SIZE OF BONES IN NORMAL AND CASTRATED[1] GUINEA PIGS

Bone	Normal, cm.	Castrate, cm.	Bone	Normal, cm.	Castrate, cm.
Head			*Pelvis*		
Total length	6.79	6.55	Innominate length	5.52	5.31
Temporal, length	2.44	2.36	Acetabulopubic length	1.43	1.43
Frontal, length	2.24	2.16	Acetabuloischial length	1.98	1.92
Nasal, length	2.25	2.19	Acetabuloilial length	3.52	3.42
Bifrontal, length	1.34	1.30	Obturator foramen	1.43	1.43
Orbit, longest axis	1.93	1.96			
Ramus of mandible, length	5.18	5.10	*Extremities*		
			Humerus, length	3.91	3.78
Back			Radioulnar, length	4.15	4.19
Scapula, length	3.68	3.54	Femur, length	4.58	4.45
Scapula, width	2.26	2.09	Tibiofibula, length	4.97	4.87
Scapula spine, width	1.04	1.01			

[1] Castrated at 3–4 months of age, autopsy 27 weeks later. Measurements made with calipers. Left and right members included in the averages

The specific role of the androgens on muscular development suggests that other hormones or factors may have a general or specific influence in the development of the muscles. It is well known that both hypophysectomy and thyroidectomy markedly retard growth.

The changes in muscle mass apparently are true changes for a proportionate change in protein (nitrogen) and water occurs and is in agreement with previous observations (Kochakian, Humm and Bartlett, 1948) on the temporal muscle. The loss in muscle mass, however, does not seem to affect the activity of the specific muscle. Thus, even though the muscles of the head are markedly retarded in growth as a result of castration, the animals seem to be able to carry on the necessary ingestion and mastication of food. It would seem that the muscle protein formed under the stimulus of the endocrine secretion of the testis must be for another and special function or else the animals make a compensatory adaptation in the intermediary biochemical processes in the smaller muscle. Scow and Roe (1953) have noted that the total amount of myoglobin in the masticatory muscles is not decreased after castration.

The difference in growth rate of the guinea pigs conforms with the difference in the total muscle mass which, at the maximum change (after 27 weeks of castration), was 37 gm. The failure to note a change in the size of the various bones after castration also conforms with the growth rate of the animals. The lack of a difference between the two groups of guinea pigs may be due to the fact that the castration was performed after the endocrine function of the testis had been well underway. On the other hand, it might be a species phenomenon. It is well known, however, that in man as well as in many domestic and laboratory animal species, striking changes occur in skeletal growth when castration is performed before puberty but these changes do not occur when castration is performed later.

The failure of the thymus to increase in size until 27 weeks after castration is of particular interest for in the rat and mouse this organ responds almost immediately after castration by an increase in size. The phenomenon in the guinea pig may be associated with age. When castration was performed 21 weeks later, an increase occurred within 6 weeks, which was equal to that produced after 27 weeks in the earlier castrated animals. It is noteworthy that the kidney showed no change in size even after 27 weeks (cf. Kochakian, Humm and Bartlett, 1948).

SUMMARY

Male guinea pigs of the inbred Hartley strain were castrated at 3–4 months of age. A group of normal animals were killed at the time of castration and paired groups of normal and castrated guinea pigs at intervals of 6, 12 and 27 weeks after castration. In addition, a group of guinea pigs was castrated 21 weeks later than the first group and killed at the

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same time as the 27-weeks groups. The internal organs and 49 individual muscles were studied. Castration had a variable effect on the muscles: (1) immediate cessation of growth with minor regression in some instances When castration was performed in the adult animals, marked regression in the mass of these muscles occurred, (2) continued growth but at a slower rate for variable periods and (3) no significant effect. The muscles of the head and neck region and the retractor penis were affected to the greatest degrees. Other muscles also showed marked changes, e.g., psoas major, pectorals and certain muscles of the extremities. The heart decreased and the adrenals increased slightly after castration. The thymus increased only in the oldest castrates. The accessory sex organs immediately regressed after castration. The other organs showed no changes.

The growth rate of the castrated animals was only slightly retarded. The nitrogen (protein) and water concentrations were not altered even in those muscles which showed great changes in mass. The various parts of the skeleton also were not changed due possibly to the fact that castration was performed after puberty.

REFERENCES

EISENBERG, E., G. S. GORDAN AND H. W. ELLIOTT: *Endocrinology* **45**: 113. 1949.
KOCHAKIAN, C. D., J. H. HUMM AND M. N. BARTLETT: *Am. J. Physiol.* **155**: 242. 1948.
PAPANICOLAOU, G. N. AND E. A. FALK: *Science* **87**: 238. 1938.
SCOW, R. O. AND J. H. ROE, JR.: *Am. J. Physiol.* **173**: 22. 1953.
WAINMAN, P. AND G. C. SHIPOUNOFF: *Endocrinology* **29**: 975. 1941.

EFFECT OF ESTRUS AND ESTROGEN ON THYROID UPTAKE OF I^{131} IN RATS[1,2]

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HYPERTHYROIDISM, nodular goiter and other types of thyroid disease are more frequently found in women than in men. Goiter, also, often becomes strikingly manifest during puberty, pregnancy and the menopause. This apparent relationship between ovarian function and thyroid pathology has been studied by many investigators. Unfortunately the results have been conflicting. Some have reported that ovarian hormones depress thyroid function (Franck, 1937; Gardner, 1949; Heyl *et al.*, 1934; Karp and Kostkiewicz, 1934; Kreitmar and Sieckman, 1939; Müller and Aeppli, 1949); others, that ovarian hormones increase thyroid activity (Andersen, 1935; Chouke *et al.*, 1935; De Amilbia *et al.*, 1936; Desclin and Ermans, 1951); and still others have observed no distinct influence upon thyroid structure and physiology (Arnold *et al.*, 1938; Paschkis *et al.* 1948). A few reports have indicated that estrogens both stimulate and depress thyroid function, depending upon the dose and period of administration (Freudenberger and Clausen, 1937a, 1937b; Tagliaferro, 1933; Wolterink *et al.*, 1950).

Over the years the hypothesis has been slowly developed that the female gonads and their hormones affect thyroid morphology and physiology via the pituitary, i.e. that estrogens induce an increased production of hypophyseal thyrotrophin with consequent effects on the thyroid (Aron and Benoit, 1932; Desclin and Ermans, 1950, 1951; Heyl *et al.*, 1934; Loeser, 1934, 1935; Franck, 1937; Lerman, 1950; Marine 1935). No evidence, however, has been offered for this assumption. Grumbrecht and coworkers (1938a, 1938b, 1939) have assayed the pituitary for TSH following castration or estrogen treatment in rats. Their observations of the action of estrogen on hypophyseal TSH were conflicting and incomplete. Turner and Cupps (1940) reported that estrogen maintained the normal hypophyseal content of TSH in gonadectomized female rats. In any case, assay

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of the quantity of TSH in the pituitary was no index of the rate of production.

The present investigation was undertaken to study the influence of the ovary and its hormones on thyroid function and structure. As part of this program the experiments to be presented were initiated to determine the effect on I^{131} uptake by the thyroid of the estrus cycle and of estrogen given to female, male and castrate male rats. In addition, the effect of estrogen on I^{131} uptake by the thyroids of hypophysectomized rats was studied. It was found that the ovarian hormone markedly increased thyroid uptake in both intact, castrate, and hypophysectomized animals.

MATERIALS AND METHODS

Mature female (Carworth) and male rats (Holtzman) were maintained on tap water and a pellet diet containing 0.43 mg. potassium iodide per 100 gm. of food.[3] Vaginal smears were made daily at the same time of the day on all female rats for at least 4 estrus cycles before an experiment and throughout the period of observation. Rats with irregular cycles were not used. Hypophysectomized female rats were obtained from Hormone Assay Laboratories, Chicago, Illinois. Both castrate and hypophysectomized animals were used 13–23 days after operation.

I^{131} was obtained from the Oak Ridge National Laboratory, Oak Ridge, Tennessee. After appropriate dilution with saline the desired microcurie dose, contained in 0.5 ml., was administered intraperitoneally.

Two or more standards, appropriately diluted, were taken for each experiment from the same stock solution that was injected into the rats and were counted at the same time and under the same conditions of geometry as were the rat thyroids. For counting a well type thallium activated gamma crystal protected by a 7.8 cm. thick lead shield with a 19 mm. opening was used.

In vivo counting was accomplished by holding unanesthetized rats over the lead shield opening at a constant distance of 10 cm. from the crystal. Two or more counts for one minute each were made at a counting rate such that the sampling error was 2% or less. Under these conditions 1 μc. of I^{131} yielded 2×10^4 counts per minute. Body background was determined by counting the region of the left shoulder. Thyroid counts were calculated, after coincidence and background corrections, by subtracting body background counts from the counts observed over the thyroid region. No further corrections were made for body contour or circulating I^{131} (Wolff, 1951).

For *in vitro* counting, rats were anesthetized with ether and exsanguinated. Their thyroids were immediately dissected out, cleaned of adventitial tissue, weighed on a Roller-Smith torsion balance to the nearest 0.1 mg., and placed in test tubes containing 0.5 ml. of saline or fixative. They were then counted in the well of the crystal counter. Under these conditions 1 μc. of I^{131} yielded 1×10^6 counts per minute. Standards were counted under the same conditions of geometry and at the same time.

For urinary and fecal excretion studies, rats were placed in individual metabolism cages. The collection bottles were washed in a 0.5% solution of potassium iodide. After a 24-hour collection the cages were washed with potassium iodide solution into the urine bottles. The quantity of fluid collected was measured to the nearest 0.5 ml. and an aliquot was taken for counting. The feces were put in 50 ml. test tubes and counted in the well of a Geiger-Müller counter having a sensitivity 1/15 that of the gamma crystal.

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[3] Allied Mills, Inc., Wayne Lab-Blox (Mouse Diet) 0.00043% Potassium Iodide.

Thyroid uptake was calculated as the percent of the injected dose after all necessary corrections had been made.

Estrogen[4] was administered subcutaneously in 0.2 ml. quantities after appropriate dilution with saline. Control rats received 0.2 ml. of saline subcutaneously.

RESULTS

I. Female Rats

a) *Effect of the Estrus Cycle on Thyroid Uptake of I^{131}.* Fifteen rats, weighing 180–200 gm., were used. Eight were in estrus and 7 were in diestrus at the time 1.2 μc. of I^{131} were injected. *In vivo* thyroid counting was done at 6, 24, 48 and 72 hours after I^{131} administration. In Table 1

TABLE 1. EFFECT OF ESTRUS ON THYROID UPTAKE OF I^{131}

Animal	6 hours	24 hours	48 hours	72 hours
estrus (8)	9.3 ± 0.90	11.8 ± 0.92	10.7 ± 0.87	8.3 ± 0.98
diestrus (7)	5.0 ± 0.45	6.4 ± 0.66	5.4 ± 0.49	4.2 ± 0.40

Results are expressed as percentages of doses injected ± standard error of mean.

thyroid radioactivity at all time intervals was highly significantly greater in estrous rats as compared with diestrous rats.

Six weeks later the same group of rats was used again after radioactivity was no longer detectable over the thyroid area. This time the I^{131} was administered to the 7 rats in estrus which had been in diestrus during the first experiment and to the 8 rats in diestrus which had previously been in estrus. *In vivo* counting was done 24 hours after injection of 1.2 μc. of I^{131}. The average uptake of the 7 rats in estrus was now 10.9 ± 0.63 (S.E.) as compared with the average uptake in 8 diestrous animals of 5.3 ± 0.47 (S.E.). The difference was highly significant ($P < 0.01$).

b) *Effect of Estrogen on Thyroidal Uptake of I^{131}.* *In vivo* thyroid uptake was measured in 53 female rats, weighing 200–250 gm., 24 hours after the administration of 1.2 μc. of I^{131}. Eleven were given a single dose of 500 I.U. of estrogen 24 hours before the iodine was injected. Eighteen were given 100 I.U. of estrogen daily for 20 days (total, 2000 I.U.). On the day

TABLE 2. EFFECT OF ESTROGEN ON THYROID UPTAKE AND EXCRETION OF I^{131} IN FEMALE RATS

Animal	Number	Uptake*	Renal excretion of I^{131}, % of dose*
Estrogen 500 I.U.	11	13.6 ± 1.43[a]	61.5 ± 3.0
Estrogen 2000 I.U. over 20 days	18	10.2 ± 0.67[b]	63.5 ± 3.5
Controls	24	7.7 ± 0.77[c]	60.1 ± 4.9

Significance:
[a] & [b] P is 0.05 to 0.02
[b] & [c] P is 0.02 to 0.01
[a] & [c] $P < 0.01$
* Results are expressed as percentage of dose injected ± standard error of mean.

[4] Theelin, aqueous (Estrone), Parke, Davis, containing 50,000 I.U. per mg.

of the last estrogen injection I^{131} was injected. Twenty-four rats comprised a control group. The results are shown in Table 2.

II. Effect of Estrogen on Thyroidal Uptake of I^{131} in Intact Male Rats

To eliminate the variations induced by the estrus cycle it was decided to use male rats as a test animal.

a) Eight male rats were given 500 I.U. of estrogen daily for 15 days (total, 7500 I.U.) and 8 controls received 0.2 ml. of saline daily over the same period. One hour after the last injection, all rats were administered approximately 2 μc. of I^{131} and were sacrificed 24 hours later.

b) Nine experimental and nine control male rats were treated precisely as above except that they were sacrificed 1 to $1\frac{1}{2}$ hours following the injection of I^{131}.

c) Nine rats received 500 I.U. of estrogen daily for 3 days (total, 1500

TABLE 3. EFFECT OF ESTROGEN ON THYROID UPTAKE OF I^{131} IN INTACT MALE RATS

Treatment	Body wt., gm.	Thyroid wt., mg.	Uptake per thyroid, % of dose injected	Uptake per 100 mg. of thyroid tissue, % of dose injected	Serum I^{131} Counts/m per 0.1 ml.
a) Estrogen 7500 I.U. over 15 days	285	18.5 ± 0.71	14.3 ± 1.11	77.6 ± 4.9	
Control	312	15.5 ± 0.74	12.4 ± 1.03	79.4 ± 5.4	
b) Estrogen 7500 I.U. over 15 days	220	12.5 ± 0.52	1.8 ± 0.14	15.4 ± 0.89	967 ± 48.7
Control	293	12.0 ± 0.75	1.8 ± 0.15	14.7 ± 0.87	792 ± 41.4
c) Estrogen 1500 I.U. over 3 days	308	11.8 ± 0.54	4.46 ± 0.41	37.0 ± 3.64	1602 ± 82.3
Control	277	12.2 ± 0.59	2.65 ± 0.41	21.0 ± 1.34	1359 ± 45.3

Results are presented with standard error of mean.

I.U.) and 9 controls 0.2 ml. of saline daily for the same period. One hour after the last injection all animals were given 2.2 μc. of I^{131} and were sacrificed 1 to $1\frac{1}{2}$ hours later.

The recorded body weight was the body weight at the time of sacrifice. All animals were between 200–250 gm. at the start of the experiment.

Table 3 presents the data of these experiments.

III. Effect of Estrogen on Thyroidal Uptake of I^{131} in Castrate Male Rats

Castrate male rats were used as a test animal to eliminate any possible influence of testicular hormones on the effect of estrogen or thyroid function.

a) Thirty-two male rats, weighing 200–250 gm. at the start of the experiment, were grouped as follows: 10 unoperated controls; 12 castrate; 10 castrate which were given 100 I.U. of estrogen daily for 3 days prior to I^{131} administration. Castration was performed 17 days before I^{131} injection. All rats received about 4 μc. of I^{131}. Twenty-four hours later *in vivo* counting of the thyroid was done. Table 4 shows the results.

TABLE 4. THYROID UPTAKE OF I^{131} IN INTACT, CASTRATE AND ESTROGEN TREATED CASTRATE MALE RATS

Treatment	Number	Body wt., gm.	Uptake, % of dose injected
Intact Controls	10	298	$5.2 \pm 0.58^{*a}$
Castrate	12	306	6.5 ± 0.33^{b}
Castrate, 300 I.U. estrogen over 3 days	10	278	9.5 ± 0.67^{c}

* Standard error of mean.
Significance:
[a] & [b] P 0.1 to 0.05
[b] & [c] P <0.01
[a] & [c] P <0.01

b) Twenty male rats, weighing 200–250 gm., were castrated 48 days before the injection of I^{131}. They were grouped as follows: 5 received 7500 I.U. of estrogen daily for 3 days; 5 received 500 I.U. of estrogen daily for 3 days; 4 received 500 I.U. of estrogen daily for 34 days; and 4 were controls. All animals were injected with 1 μc. of I^{131} on the day of the final estrogen injection and sacrificed 24 hours later. The data are presented in Table 5.

IV. Effect of Estrogen on Thyroidal Uptake of I^{131} in Hypophysectomized Female Rats

a) Twelve hypophysectomized female rats, 13 days post-hypophysectomy, were injected with approximately 10 μc. of I^{131}. They were put in individual metabolism cages. Twenty-four hours later I^{131} activity over the thyroid and in the urine and feces was recorded.

Five weeks later the same rats were given 50 I.U. of estrogen daily for 6 days. On the day of the last injection, all animals received 5 μc. of I^{131} and were placed in metabolism cages. Twenty-four hours later thyroid, urine and feces were counted. Just prior to the second injection of I^{131}, the thyroid

TABLE 5. EFFECT OF ESTROGEN ON THYROID UPTAKE AND EXCRETION OF I^{131} IN CASTRATE MALE RATS

Treatment	Number	Body wt., gm.	Thyroid wt., mg.,	Uptake per gland	Uptake per 100 mg. thyroid tissue	Renal excretion of I^{131}, % of dose
Estrogen, 22,500 I.U. over 3 days	5	307	$13.2 \pm 0.85^{*}$	$6.8 \pm 1.13^{*}$	$52.3 \pm 10.1^{*a}$	$62.0 \pm 4.5^{*}$
Estrogen, 1500 I.U. over 3 days	5	312	15.7 ± 1.11	10.5 ± 1.57	76.4 ± 12.0^{b}	50.9 ± 6.2
Estrogen, 17,000 I.U. over 34 days	4	262	12.8 ± 0.42	1.6 ± 0.30	13.1 ± 2.2^{c}	37.1 ± 7.3
Control Castrates	4	352	15.4 ± 0.79	6.1 ± 1.06	39.7 ± 7.5^{d}	70.8 ± 3.8

* Standard error of mean.
[a] is not significantly different from [b] and [d].
[b] and [d] are significantly different at .05% level.
[c] is significantly lower than all other groups.

TABLE 6. EFFECT OF ESTROGEN ON I^{131} UPTAKE AND EXCRETION IN HYPOPHYSECTOMIZED RATS

Animal	Body wt., gm.	Thyroid uptake %	Urine excretion %	Fecal excretion %
Control period	134	0.6 ± 0.07	44.3 ± 1.0	1.5 ± 0.48
Estrogen 50 I.U. over 6 days	133	1.4 ± 0.18	45.8 ± 1.6	1.9 ± 0.99

Results are expressed as per cent of dose injected ± standard error of mean.

area of all rats was counted for residual activity and this was subtracted from subsequent counts. Residual activity averaged 5% of the counts recorded 6 weeks previously.

The results of this experiment are presented in Table 6.

b) In the previous experiment each animal was its own control and received two injections of I^{131} six weeks apart. To eliminate any possible damaging effects of I^{131} over a period of six weeks and also the residual activity of the first injection, a second experiment was run using 21 hypophysectomized rats. Seven received 100 I.U. of estrogen daily for 3 days and 14 received 0.2 ml. of saline. One hour after the last injection, all rats were given 23 μc. of I^{131} (21 days post-hypophysectomy). Twenty-four hours later *in vivo* counting of the thyroid area was accomplished. The results are shown in Table 7.

V. Effect of Varying Doses of Estrogen on Thyroidal Uptake of I^{131} in Hypophysectomized Rats

Eighteen rats were grouped as follows: 5 received 5000 I.U. of estrogen daily for 2 days; 5 received 100 I.U. of estrogen daily for 2 days; 5 received 500 I.U. of estrogen daily for 21 days; 3 comprised a control group. On the last day of estrogen injections, 23 days post-hypophysectomy, all animals were given approximately 3 μc. of I^{131}. They were placed in individual metabolism cages for urine collection and sacrificed 24 hours later. Table 8 contains the results of this experiment.

VI. Effect of Estrogen on Thyroidal Uptake of I^{131} in Hypophysectomized Rats Treated With TSH

Eleven rats were injected with 5 mg. of U.S.P. thyrotrophin (0.25 units) twice daily for 3 days and once in the morning of the fourth day. Concomitantly 6 of the 11 rats received 100 I.U. of estrogen once daily for

TABLE 7. EFFECT OF ESTROGEN ON I^{131} UPTAKE IN HYPOPHYSECTOMIZED RATS

Animal	Number	Body wt., gm.	Uptake, % of injected dose
Estrogen 100 I.U. over 3 days	7	127	1.92 ± 0.22*
Controls	14	140	1.00 ± 0.15

* Standard error of mean.

TABLE 8. EFFECT OF VARYING DOSES OF ESTROGEN ON I^{131} UPTAKE IN HYPOPHYSECTOMIZED RATS

Animal	Number	Body wt., gm.	Thyroid wt., mg.	Uptake* per gland %	Uptake* per 100 mg. of thyroid %	Urine* excretion %
Estrogen 5000 I.U. for 2 days	5	113	7.7 ± 0.64	0.78	10.3 ± 0.87	40.7 ± 6.7
Estrogen 100 I.U. for 2 days	5	116	7.0 ± 0.30	0.22	3.2 ± 0.36	49.2 ± 6.7
Estrogen 500 I.U. for 21 days	5	107	7.2 ± 0.44	0.21	2.9 ± 0.33	38.0 ± 3.0
Controls	3	120	8.2 ± 1.3	0.10	1.2 ± 0.31	55.5 ± 3.2

* Uptake and excretion results are expressed as percentage of dose injected ± standard error of mean.

3 days and 5 controls received 0.1 ml. of saline over the same period. One hour after the last injection of thyrotrophin, all animals were given 0.6 μc. of I^{131} and sacrificed 1½ hours later (21 days post-hypophysectomy). The observations are shown in Table 9.

TABLE 9. EFFECT OF ESTROGEN ON THYROIDAL UPTAKE OF I^{131} IN HYPOPHYSECTOMIZED RATS TREATED WITH TSH

Animal	Number	Body wt., mg.	Thyroid wt., mg.	Thyroid uptake per gland, % of dose injected	Thyroid uptake per 100 mg. of thyroid, % of dose injected
Estrogen 100 I.U. over 3 days	6	129	8.0 ± 0.35*	5.4 ± 0.53*	68.0 ± 10.4*
TSH Controls	5	139	8.2 ± 0.81	4.5 ± 0.34	53.7 ± 11.4

* Standard error of mean.

DISCUSSION

The experiments reported here clearly demonstrated that estrus and estrogen given for short periods of time significantly increased the thyroid uptake of I^{131} in female, male and male castrate rats. This had been described previously by Soliman (1952) and Soliman and Reineke (1954). The increased uptake was effected by a wide dose range of estrogen given over a short period of time, i.e., 1 to 3 days. Doses varied from 100 I.U. to 7500 I.U. of estrogen daily or about 5 to 500 times the estimated physiologic dose (Greep and Jones, 1950). The I^{131} uptake was increased by estrogen or during estrus 50 to 100% above the uptake observed in control animals. No apparent dose-response correlation was noted, which indicated a thyroid response independent of size of dose over a short period of time.

Prolonged injection of estrogen, i.e., 15 days or more, elicited equivocal results. In female rats given 100 I.U. daily for 20 days the I^{131} uptake was significantly greater than in non-treated control rats, but less than in females given a single injection of estrogen. In male animals given 500 I.U.

daily for 15 days the uptake was the same as in control rats. In castrate males given 500 I.U. daily for 34 days the uptake was significantly lower than in untreated controls or in castrates which had received equivalent doses for only 3 days. These data indicated that prolonged administration of estrogen prevented the increased uptake of I^{131} or even depressed the thyroid uptake. Tagliaferro (1933) reached similar conclusions based upon histological changes in the thyroid. He reported hyperplasia of the thyroid after 5 to 10 days of estrogen treatment in rats and guinea pigs, and involution after 30 days of estrogen administration. Soliman (1952) also reported that small doses of estrogen over a short period increased significantly thyroid uptake of I^{131} but large doses of estrogen over a longer period did not affect thyroid uptake. Comparable conclusions on the stimulatory and inhibitory actions of estrogen on thyroid structure and function have been reached by Freudenberger and Clausen (1937a, 1937b) and by Wolterink *et al.* (1950).

Of greater interest, similar results were obtained in hypophysectomized rats and demonstrated that the effect of estrogen on thyroid uptake of I^{131} was not mediated by the pituitary. After a few days of estrogen injections I^{131} uptake was always markedly increased; after 21 days of estrogen injections the I^{131} uptake was significantly elevated above control values but was significantly less than in animals receiving an equivalent dose over 2 days. It has been assumed that the structural and functional changes in the thyroid elicited by castration or estrogen treatment were due to alterations of hypophyseal thyrotrophin (Aron and Benoit, 1932; Desclin and Ermans, 1950, 1951; Franck, 1937; Gassner, 1948, Lerman, 1950; Marine, 1935). Very little concrete evidence has been marshalled to prove this assumption. Grumbrecht and his co-workers (1938a, 1938b, 1939) have performed TSH assays of pituitaries from animals treated with estrogen or from castrates. They have reported thyroid hyperplasia associated with diminished TSH content in the pituitary after castration or estrogen treatment. Their method of assay, however, was poor and yielded no information about hypophyseal production and discharge of TSH. Turner and Cupps (1940) have described diminished hypophyseal content of TSH following castration in rats and restoration to normal values by estrogen injections. Again, the method did not reveal the quantity produced and discharged into the circulating blood. The experiments reported in this paper have not ruled out a possible estrogen effect upon hypophyseal TSH. They have shown, however, that estrogen could influence the thyroid without the mediation of the pituitary.

The variation in thyroid reponse to brief and prolonged estrogen-treatment may explain some of the conflicting observations recorded previously. Different doses of estrogens administered over varying periods of time have been reported to cause thyroid enlargement (Desclin and Ermans, 1950; Freudenberger and Clausen, 1937; Leiby, 1933; Pincus and Werthessen,

1933), hyperplasia (DeAmilbia *et al.*, 1936; Desclin and Ermans, 1951), proliferation (Chouke *et al.*, 1935), no change (Arnold *et al.*, 1938; Schilling *et al.*, 1941), involution and atrophy (Franck, 1937; Gardner, 1949; Heyl *et al.*, 1934; Karp and Kostkiewicz, 1934; Kreitmar and Sieckman, 1939), and necrosis and fibrosis (Morrell and Hart, 1941). Employing the technique of I^{131} uptake Paschkis *et al.* (1948) observed no effect following estrogen injections, Müller and Aeppli (1949) described a decreased uptake and Money *et al.* (1950) reported both increased and decreased uptakes using different estrogens.

No significant influence of estrogen on absolute thyroid weight was noted. In some instances, however, the administration of estrogen was accompanied by significant differences in thyroid weight when comparisons were made on the basis of mg. of thyroid per 100 gm. of body weight. This may also explain some of the conflicting reports on thyroid alterations due to estrogen.

Estrogen did not inhibit nor potentiate the action of TSH on thyroid uptake of I^{131}. Aron and Benoit (1932) and Gessler (1937) reported that estrogen inhibited or neutralized the action of TSH on the thyroid. Desclin and Ermans (1951) and Heyl *et al.* (1934) were unable to detect any effect of estrogen on TSH activity. The original intention of the experiment was to learn whether estrogen might enhance the action of TSH on the thyroid. Apparently the numerous changes in thyroid physiology induced by TSH masked or prevented the estrogen effect.

The precise locus of action of estrogen has not been determined by the studies presented here. Presumably estrogen affected the thyroid directly. There is, however, an alternative possibility which might explain the present data. An increased iodide concentration in the sera of estrogen treated animals would permit the thyroid to extract more iodide from the blood than it could remove from the blood of control animals. In Table 3 is recorded the radioactivity of sera collected from experimental and control rats one and one-half hours after the administration of I^{131}. The data indicated that the iodide concentration in the sera of estrogen treated rats was up to 20% greater than the iodide concentration in the sera of control rats. (It is most likely that there is little or no protein bound I^{131} in the sera one and one-half hours after I^{131} injection.) The differences in sera radioactivity were by themselves insufficiently great to account for the 75% greater thyroidal uptake in rats treated briefly with estrogen. This situation has been noted in other groups of rats, the data of which are not recorded here.

Diminished renal excretion of iodide with an elevated serum iodide concentration might be expected to result in an increased thyroidal uptake of I^{131}. In these experiments (Tables 2, 5, 6 and 8) renal excretion of I^{131} was slightly diminished in some instances by the administration of estrogen, and significantly so when the ovarian hormone was injected over a pro-

longed period of time. Since the thyroid and kidneys compete for iodide, these results suggested that more iodide might have been available to the thyroid in estrogen treated animals. However, in those rats with the greatest diminution of excreted I^{131} (Tables 5 and 8), the thyroidal uptake was less than or only slightly more than control values. The effect of estrogen on renal excretion of I^{131} must await further quantitative studies on serum and urinary levels of both I^{127} and I^{131}.

SUMMARY

Thyroid uptake of I^{131} was increased during estrus. Administration of estrogen for short periods of time to intact female and male, castrate male and hypophysectomized female rats induced an increased I^{131} uptake by the thyroid. The response of the thyroid was independent of dose of estrogen over short periods of time. Prolonged injection of estrogen prevented the increased uptake of I^{131} or depressed its usual uptake. The administration of estrogen did not affect thyroid weight. The precise site of action of estrogen was not determined but its effect was not mediated by the pituitary.

REFERENCES

ANDERSEN, D. H.: *J. Physiol.* **83**: 15. 1935.
ARON, M. AND J. BENOIT: *Compt. Rend. de la Soc. de Biol.*, **109**: 923. 1932.
ARNOLD, O. H., P. GRUMBRECHT AND A. LOESER: *Arch. f. Exptl. Path. u. Pharmak.* **191**: 192. 1938a.
CHOUKE, K. S., H. FRIEDMAN AND L. LOEB: *Anat. Rec.* **63**: 131. 1935.
DEAMILBIA, E., M. M. MENDIZOBAL AND J. BOTELLA-LLUSIA: *Klin. Wchnschr.* **15**: 1001. 1936.
DESCLIN, L. AND A. M. ERMANS: *Compt. Rend. de la Soc. de Biol.* **144**: 1277. 1950.
DESCLIN, L. AND A. M. ERMANS: *Ann. Endocrinol.* **12**: 238. 1951.
FRANCK, S.: *Compt. Rend. de la Soc. de Biol.* **125**: 573. 1937.
FREUDENBERGER, C. B. AND F. W. CLAUSEN: *Anat. Rec.* **68**: 133. 1937a.
FREUDENBERGER, C. B. AND F. W. CLAUSEN: *Anat. Rec.* **69**: 171. 1937b.
GARDNER, J. H.: *Proc. Soc. Exp. Biol. & Med.* **72**: 306. 1949.
GASSNER, F. X., H. W. BARRET AND R. G. GUSTAVSON: *West. J. Obs. Surg. & Gyn.* **56**: 346. 1948.
GESSLER, C.: *Arch. Intl. de Pharm. et de Ther.* **55**: 267. 1937.
GREEP, R. O. AND I. C. JONES: *Recent Progress in Hormone Research* Vol. **V**: 197. 1950
GRUMBRECHT, P. AND A. LOESER: *Arch. f. Exptl. Path. u. Pharmak.* **189**: 345. 1938b.
GRUMBRECHT, P. AND A. LOESER: *Arch. f. Exptl. Path. u. Pharmak.* **192**: 202. 1939.
HEYL, J. G., S. E. DEJONGH AND R. KOOY: *Acta Brevia Neerland* **4**: 126. 1934.
KARP, L. AND B. KOSTKIEWICZ: *Klin. Wchnschr.* **13**: 489. 1934.
KREITMAR, H. AND W. SIECKMAN: *Klin. Wchnschr.* **18**: 156. 1939.
LEIBY, G. M.: *Proc. Soc. Expt. Biol. & Med.* **31**: 15. 1933.
LERMAN, J.: *Progress in Gynecology* **11**: 253. 1950.
LOESER, A.: *Klin. Wchnschr.* **13**: 766. 1934.
LOESER, A.: *Klin. Wchnschr.* **14**: 4. 1935.
MARINE, D.: *J.A.M.A.* **104**: 2250. 1935.

UNIVERSITY OF MICHIGAN LIBRARIES

MONEY, L., L. KIRSCHNER, L. KRAINTZ, P. MERRILL AND R. RAWSON: *J. Clin. Endocrin.* **10:** 1282. 1950.
MORRELL, J. A. AND G. W. HART: *Endocrinology* **29:** 995. 1941.
MÜLLER, J. H. AND H. AEPPLI: *Gynecologia* **128:** 422. 1949.
PASCHKIS, K. E., A. CANTAROW AND W. C. PEACOCK: *Proc. Soc. Exp. Biol. & Med.* **68:** 485. 1948.
PINCUS, G. AND N. WERTHESSEN: *Am. J. Physiol.* **103:** 631. 1933.
SCHILLING, W. AND G. L. LAQUEUR: *Endocrinology* **29:** 103. 1941.
SOLIMAN, F. A.: 1952. Thesis submitted to School of Graduate Studies, Michigan State College of Agriculture and Applied Sciences.
SOLIMAN, F. A. AND E. P. REINEKE: *Am. J. Physiol.* **178:** 89. 1954.
TAGLIAFERRO, P.: *Folia Gynaec.* **30:** 597. 1933.
TURNER, C. W. AND P. T. CUPPS: *Endocrinology*, **26:** 1042. 1940.
WOLFF, J.: *Endocrinology* **48:** 284. 1951.
WOLTERINK, L. F., C. C. LEE, K. OLSEN AND M. MURRAY: *Fed. Proc.* **9:** 138. 1950.

INFLUENCE OF DIETHYLSTILBESTROL ON LIPIDS IN INTACT AND HYPOPHYSECTOMIZED COCKERELS[1]

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THERE is ample evidence to show that the remarkably high level of blood lipids attained by laying hens and other birds is associated with hormones elaborated by the developing follicles. Much of the early literature has been reviewed by Riddle (1927, 1942).

The effectiveness of estrogens in simulating in immature pullets and cockerels the concentration of blood lipids characteristic of laying hens was demonstrated in a series of publications by Entenman, *et al.* (1938, 1940) and Lorenz, *et al.* (1939). Other steroid hormones such as androgen, progesterone, and desoxycorticosterone do not similarly affect blood lipids.

Restriction of dietary fat (Horlock and Katz, 1948) or starvation (Zondek, 1941) does not prevent the elevation of blood lipids in estrogen-treated cockerels. Observations by Bird (1946) further indicate that estrogen does not promote more efficient absorption of fat from the gut. The source of lipids in the bloodstream following estrogen treatment, therefore, is not exogenous fat.

Prolonged treatment of cockerels with estrogen causes not only a sustained lipemia but also increased deposition of fat in the tissues and organs (Lorenz, 1943; Stamler, *et al.*, 1950). This evidence indicates that the blood lipids are not derived from the mobilized depots but are caused by the increased synthesis of lipids, perhaps in conjunction with a normal or decreased utilization and deposition of lipids. Treatment of cockerels with diethylstilbestrol enhances the *in vitro* formation of phospholipids by the liver (Taurog, *et al.*, 1944), while functional hepatectomy of cockerels prevents the appearance of additional blood lipids following estrogen treatment (Ranney, *et al.*, 1951).

The means by which estrogen affects lipid metabolism remains speculative. Riddle and Senum (1939) have stated that estrogen induces lipemia in hypophysectomized as well as intact pigeons. Nalbandov and Card (1943) have reported similar findings in chickens. Zondek (1941) failed to prevent lipemia in intact cockerels by administering whole anterior pituitary, thyrotrophin or corticotrophin along with estrogen.

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The following study is an attempt to further elucidate the mechanism influenced by estrogen which results in lipemia and increased fat deposition in cockerels.

MATERIALS AND METHODS

Gray Leghorn cockerels were obtained as day-old chicks from Sunnyside Hatchery, Madison, Wisconsin. They were kept in an electrically heated brooder for the first three weeks and fed a commercial chick starter. Subsequently they were moved to standard chick batteries and the chick starter was replaced by a commercial adult ration. Food and water were available at all times. Birds were placed on experiment or hypophysectomized at nine or ten weeks of age at which time they weighed approximately 850–1000 gm. Those which were hypophysectomized were allowed a recovery period of a week or ten days before being placed on experiment.

It was found desirable to force-feed a standard amount to all experimental birds because on an *ad libitum* regime food intake is variable. A synthetic diet consisting of 65% sucrose, 18% casein, 5% gelatin, 5% corn oil, 6% Salts V (Briggs, 1943), and recommended amounts of vitamins was fed. A week's supply of diet was mixed at one time and stored in a metal container at 40° F. For feeding, 30 gm. of the mixture was made to a volume of 55 ml. with water, yielding a suspension which could be easily fed with a 50 ml. volumetric pipette fitted with a rubber bulb at one end and a short length of rubber tubing at the other. Two feedings a day were given—one at 8 A.M. and the other at 4–4:30 P.M. The weekly gains of birds fed in this manner were found to be similar to those of birds allowed to feed *ad libitum*. Unlike rats, which must be allowed to adjust to force-feeding by gradually increasing the ration over several days, chickens suffer no ill effect from force-feeding a full ration immediately.

The fat-free diet was essentially the same as the regular diet except that the corn oil was omitted, the sucrose increased to 67% and the casein to 21%. Once a week one cc. per bird of cod liver oil fortified with alpha tocopherol was added to the ration. It has been shown that fat is not essential in the diet of growing chicks if the fat-soluble vitamins are supplied in adequate amounts (Russel, *et al.*, 1940).

Diethylstilbestrol was injected intramuscularly in 0.1 cc. of Mazola oil. Control animals were injected with 0.1 cc. of the vehicle. The experimental period was from three days to three weeks depending on the dosage of the hormone given. Force-feeding and injections were begun simultaneously in the long-term experiments. In those experiments in which there were only three days of injections, the force-feeding was begun two days prior to beginning the estrogen administration. At the end of the experimental period the birds were given an overdose of Nembutal intravenously and blood was taken from the heart immediately and placed in a heparinized centrifuge tube. The liver was weighed and samples for analysis removed and stored in glass vials at −4° C. The mass of fat around the cloaca and gizzard was removed and weighed as an index of depot fat. Thyroids, adrenals, and gonads were dissected and weighed on a Torsion balance.

Daily food intake was determined for both intact and hypophysectomized birds. These animals were kept in individual cages provided with feeders which prevented wasting of food. The weight of the feeder was recorded at the same time every day and the daily food consumption obtained by difference. Four to eight birds were observed at one time. A control period of 8–10 days was obtained for all birds. Injections of .8 mg. diethylstilbestrol per day were then begun on half of the birds, the other half being continued for control observations. Injections were continued for 10–14 days. At the end of this period the hormone was discontinued and the previous control groups were given diethylstilbestrol for a period of 10–14 days. Observations were also continued on the

TABLE 1. EFFECT OF DIETHYLSTILBESTROL ON THE DISTRIBUTION OF LIPIDS AND WEIGHT GAIN[1]

Treatment	Exp. period	Final weight, gm.	Weight gain, gm.	Visceral fat, gm.	Blood		Liver	
					Lipid PO_4 mg./100 ml.	Total lipid mg./100 ml.	Lipid PO_4 mg./100 gm.	Total lipid gm./100 gm.
Intact								
Oil (9)[2]	3 wks.	1280 ± 72	419 ± 65	14.0 ± 6.1	9.8 ± 2.0	378 ± 83	94.4 ± 6.1	4.3 ± 0.6
.25 mg. DES[3] (9)	3 wks.	1233 ± 93	379 ± 83	19.0 ± 7.1	11.7 ± 2.2	594 ± 258*	100.0 ± 5.2	5.8 ± 2.1
.4 mg. DES (3)	3 wks.	1293 ± 65	307 ± 3	20.5 ± 7.4	17.2 ± 1.4**	928 ± 103**	102.2 ± 3.2	4.9 ± 1.0
Hypophysectomized								
Oil (8)	3 wks.	1293 ± 65	397 ± 51	26.8 ± 6.1	10.5 ± 2.4	577 ± 200	97.8 ± 4.1	4.9 ± 0.7
.25 mg. DES (6)	3 wks.	1394 ± 75	503 ± 67	24.6 ± 2.6	14.8 ± 1.8**	791 ± 96*	96.8 ± 6.2	5.2 ± 1.7
Intact								
Oil (4)	3 days	950 ± 87	65 ± 24	3.3 ± 1.5	9.1 ± 0.3	465 ± 104	96.6 ± 2.1	4.1 ± 0.6
2 mg. DES (5)	3 days	916 ± 29	62 ± 16	4.2 ± 2.0	35.9 ± 7.4**	2526 ± 457**	98.8 ± 2.5	3.9 ± 1.1
Hypophysectomized								
Oil (4)	3 days	1080 ± 46	34 ± 16	9.6 ± 2.7	9.4 ± 1.6	450 ± 118	96.3 ± 4.5	4.8 ± 0.7
2 mg. DES (5)	3 days	1071 ± 153	41 ± 25	6.8 ± 2.5	30.1 ± 5.9**	2148 ± 538**	99.8 ± 8.6	4.2 ± 0.5
				Fat-Free Diet				
Intact								
Oil (9)	3 wks.	1261 ± 125	265 ± 45	3.5 ± 1.5	9.8 ± 1.3	377 ± 120	92.8 ± 7.0	3.5 ± 0.4
.4 mg. DES (11)	3 wks.	1215 ± 55	204 ± 56	10.9 ± 3.3**	25.4 ± 8.2**	2061 ± 1275**	98.2 ± 7.1	4.0 ± 0.9
Hypophysectomized								
Oil (6)	3 wks.	1429 ± 56	429 ± 69	19.6 ± 2.6	13.9 ± 2.1	798 ± 210	86.3 ± 5.1	5.3 ± 0.8
.4 mg. DES (10)	3 wks.	1534 ± 89	485 ± 94	19.1 ± 5.4	20.2 ± 5.8	1301 ± 238**	99.0 ± 14.8	4.5 ± 1.5
Oil (4)	1 wk.	1274 ± 75	74 ± 73	12.3 ± 4.0	8.3 ± 0.9	519 ± 9	72.8 ± 0.6	3.9 ± 0.9
.8 mg. DES (5)	1 wk.	1191 ± 51	134 ± 21	14.7 ± 4.3	28.9 ± 6.3**	2121 ± 455**	77.8 ± 2.9	3.3 ± 0.8

[1] Means and Standard Deviations.
[2] Number of animals.
[3] Diethylstilbestrol.
* Probability greater than .05.
** Probability greater than .01.

previously injected group. Control and experimental data were thus obtained for each bird, but a control and experimental group were always run simultaneously.

Lipid phosphate and total lipid in the blood plasma were determined according to the method of Eilert (1953). A 500 mg. sample of the frozen liver along with 4 gm. of anhydrous sodium sulfate was ground in a mortar to the consistency of a fine powder. This was then transferred quantitatively to an Erlenmeyer flask containing 80 cc. of 3 to 1 alcohol-ether, thoroughly mixed and allowed to stand overnight. Filtration and determination of lipid phosphate and total lipid in the extract were carried out by the methods used for blood plasma.

RESULTS

The results recorded in Table 1 show the effect of diethylstilbestrol on blood and tissue lipids when the caloric intake is held constant. Treated and untreated groups were compared by the Fisher-Student t test. Differences with a probability of .05 to .01 are considered significant.

Intact Birds. The most striking effect of estrogen treatment is the elevation of blood lipids. Relatively small doses of diethylstilbestrol given over a long period of time will produce significant increases in both lipid phosphate and total lipid even when essentially all fat is eliminated from the diet. With a large dose of diethylstilbestrol even more profound elevation of blood lipids can be produced in a relatively short time. Three weeks of treatment is sufficient for the hormone to exert its influence on fat depots and weight gain. More fat is present in the depots of those birds treated with diethylstilbestrol. This was most apparent in the group fed a fat-free diet in which weight gain and fat depots were low. The total weight gained by intact birds is not influenced significantly by diethylstilbestrol, but tends to be less than in untreated birds.

The concentration of lipids in the liver was not altered by diethylstilbestrol in any of the force-fed groups. This is in contrast to published reports (Lorenz, 1943; Stamler, *et al.*, 1950) and to our own results with birds fed *ad libitum*. An average total lipid value of 10.0 ± 3.8 gm.% was found in a group of nine birds implanted with 15 mg. stilbestrol pellets for five weeks. Fatty livers were observed in estrogen treated birds force-fed 90 gm. per day instead of the usual 60 gm.

Hypophysectomized Birds. Removal of the pituitary leads to an increase in blood lipids and depot fat. Birds which have been hypophysectomized two to three months may have total blood lipid values of approximately 1000 mg.%. Nevertheless, it is possible to further elevate blood lipids significantly by estrogen treatment, regardless, of the duration of hypophysectomy or amount of fat in the diet. Visceral fat depots and liver lipids were not affected by diethylstilbestrol. Weight gain tended to be improved by estrogen but not significantly so.

Organ Weights. Table 2 summarizes the organ weights obtained from the groups of birds shown in Table 1. The effect of hypophysectomy on thyroid and gonad weight is consistent with other reports. The adrenal glands atrophy only slightly after removal of the pituitary. The livers of

TABLE 2. EFFECT OF HYPOPHYSECTOMY AND DIETHYLSTILBESTROL ON ORGAN WEIGHT[1]

Treatment[3]	Liver wet wt., gm.	Liver dry wt., gm.	Liver dry wt./ body wt.	Thyroids, mg.	Adrenals, mg.	Gonads, gm.
Intact						
Oil (9)[2]	26.9±2.5	—	—	108±21	126±18	2.16 ±1.71
.25 mg. DES (9)	30.5±3.1	—	—	122±30	135±17	.342± .087
Hypophysectomized						
Oil (8)	35.0±3.6	—	—	46±14	100±20	.209± .033
.25 mg. DES (6)	39.1±4.4	—	—	33± 5	97±13	.254± .043
Intact						
Oil (4)	29.1±3.4	7.84± .82	.887±.067	77± 8	93±15	1.088±1.34
2 mg. DES (5)	30.1±2.7	8.32± .76	.910±.105	66±20	104±12	.895± .993
Hypophysectomized						
Oil (4)	29.0±1.6	7.76± .49	.721±.076	40± 3	88±10	.211± .026
2 mg. DES (5)	33.1±6.1	8.32±1.22	.777±.056	48±16	92± 8	.294± .035
			Fat-Free Diet			
Intact						
Oil (9)	27.8±3.0	7.56± .71	.605±.053	96±19	123±22	1.956±2.2
.4 mg. DES (11)	32.5±3.2	8.87± .91	.729±.063	92±15	125±40	.392± .078
Hypophysectomized						
Oil (6)	37.0±2.9	9.40± .90	.657±.044	36± 7	110±13	.188± .037
.4 mg. DES (10)	42.2±4.8	11.06±1.75	.707±.099	40± 9	117±17	.298± .054

[1] All values are for means and standard deviations.
[2] Number of animals.
[3] Length of treatment is equivalent to that for the same groups shown in Table 1.

hypophysectomized birds tend to be heavier than those of intact birds, a difference which largely disappears when the weight is expressed in terms of body weight.

Estrogen does not alter the weight of the thyroids or adrenals of either intact or hypophysectomized birds. The inhibition of gonadotrophic hormone is obvious from the atrophic testes produced by diethylstilbestrol in intact birds. Estrogen increases both wet and dry weight of the livers in both intact and hypophysectomized birds but the increase, while consistent, is not significant.

Food Intake. Table 3 illustrates the *ad libitum* food consumption associated with the four types of experimental procedures employed in this study. Diethylstilbestrol injected into intact cockerels increased food consumption by 50%. Additional amounts of pantothenic acid, thiamine, or riboflavin given by gavage daily did not prevent the increase. There are apparently no relative deficiencies of cofactors containing these vitamins and the response is presumably due to an increased caloric requirement. Rate of gain was also significantly increased by diethylstilbestrol but the

TABLE 3. FOOD INTAKE AND WEIGHT GAIN[1]

Group	Wt. gain per day (gm.)	Food consumed per day (gm.)	Food consumed 10 gm. gain (gm.)
Intact (15)[2]	21.1±2.1	100.0± 8.0	47.8± 6.2
Intact—.8 mg. DES (16)	29.2±3.3**	142.6± 7.7**	49.5± 5.1
Hypophysectomized (18)	15.0±5.2	86.6±16.3	62.1±15.9
Hypophysectomized—.8 mg. DES (11)	14.0±6.7	87.2±17.5	62.1±26.8

[1] Mean and standard deviation of all measurements, ranging from 8–29 days.
[2] Number of animals.
** Probability greater than .01.

efficiency of gain (gm. of food consumed per 10 gm. of gain) was not affected. The hypophysectomized birds consumed about 15% less food than the intact birds and the rate and efficiency of gain was considerably poorer. Estrogen did not affect food consumption, rate of gain, or efficiency of gain in the hypophysectomized birds.

DISCUSSION

The effect of diethylstilbestrol on food consumption in intact cockerels has been noted previously by Thayer, *et al.* (1944), Stamler, *et al.* (1950), and Bird (1946). The increased caloric intake permits the deposition of large amounts of fat in the adipose tissue and fatty infiltration of the liver and other tissues of estrogen-treated birds allowed free access to food. When food intake is maintained at a constant level, deposition of fat is still favored in those birds treated with diethylstilbestrol. This is also true when fat is withheld from the diet. The excessive amounts of lipids in the bloodstream together with the larger deposits of fat support the hypothesis put forth by Stamler (1950) that the synthesis of lipids is increased by estrogen while the disposal of lipids by deposition or utilization is normal or possibly reduced. Unfortunately, absolute amounts of lipids in the bloodstream and depots do not supply information concerning the rate of synthesis or destruction of these lipids. It is possible that lipids accumulate because the energy requirement of the bird is reduced, leaving more of the available energy to be stored. Another alternative is that estrogen in some way prevents the utilization of the energy stored in the form of fat.

The involvement of the thyroid gland in estrogen-induced lipemia has been postulated by a number of authors. Bird (1946) reported a decrease in basal metabolic rate as measured by heart rate in his birds treated with diethylstilbestrol. He proposed that the reduction in BMR resulted from a depressed thyroid function due to stimulation of the adrenal glands by estrogen. Hypertrophy of the adrenals of chickens and pigeons by estrogen has been reported by a number of authors (Kumaran and Turner, 1949; Breneman, 1942; Miller and Riddle, 1939; and Stamler, *et al.*, 1950). Kar (1947) reported no effect on estrogen on adrenal weight but he observed an increase in size of the vacuoles of the cells of the cortex. No adrenal hypertrophy from estrogen treatment was observed in any of the birds used in our study.

Thiouracil causes a rise in blood cholesterol due to a shift of cholesterol from the tissues, but it does not elevate any of the other lipid fractions of the blood (Fleischmann and Fried, 1945). Thyroxine will prevent the lipemia induced by estrogen without preventing the expected increase in oviduct weight (Hertz, *et al.*, 1951). Thyroxine also prevents the increase in liver weight and protein content (Common, *et al.*, 1950) which has been reported by a number of previous workers (Common, *et al.*, 1948; Clavert,

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1944). Common, *et al.* (1950) proposed that the growth of the liver is concerned with the increased synthesis of serum phosphoproteins and phospholipids which occurs following estrogen treatment. This hypothesis is supported by the findings of Taurog, *et al.* (1944) and Ranney, *et al.* (1951) which implicates the liver as the site of these synthetic processes. Further evidence that estrogen depresses thyroid function is the work of Epstein and Wolterink (1949) who found that estrogen depresses the rate of turnover of iodine in the thyroids of normal chicks and of chicks made hypothyroid by thiouracil. There is, therefore, evidence to indicate that estrogen influences the thyroid. Presumably, the resulting lower metabolic rate would require less of the caloric intake for energy purposes, leaving more to be synthesized to fat and stored in the depots.

The hypophysectomized chicken is an example of severe hypothyroidism, manifested not only by thyroid atrophy, but also by lethargic behavior, abnormal feather structure, and reduced appetite. Clearly, other deficiencies are also present but the thyroid and gonads are most atrophic and apparently produce the most obvious deficiencies present. The adrenal glands undergo only slight atrophy and the carbohydrate metabolism, as measured by blood glucose, liver glycogen, and glucose tolerance is only slightly abnormal (Baum and Meyer, 1955; Van Horn, 1955). The relatively high blood lipids and accumulation of depot fat in hypophysectomized birds probably reflect depressed thyroid function with its attendent low energy requirement.

Diethylstilbestrol is almost as effective in hypophysectomized birds as it is in intact birds with respect to elevating blood lipids. It is effective whether or not fat is included in the diet and it is effective in birds which have been without a pituitary for from ten days to three months.

It seems unlikely, therefore, the lipemia results merely from a depression of thyroid function. Since the thyroid in the hypophysectomized bird is already severely atrophic, one would have to postulate that estrogen further depresses it, which does not seem probable. To induce severe lipemia in the intact bird, estrogen would have to produce in a short period of time a thyroid deficiency more acute than exists in a bird which has been without a pituitary for several months. In addition, if a lower energy requirement were promoting accumulation of fat, one would expect the estrogen-treated intact birds to show greater weight gains than the untreated birds. Actually, weight gains are consistently less in estrogen-treated birds and a larger per cent of the gain is in the form of fat.

It does not appear that the response of hypophysectomized birds to estrogen is identical to the response of intact birds. Appetite is not stimulated by diethylstilbestrol unless the pituitary is present. There is no increased deposition of fat from estrogen treatment either when the birds are fed *ad libitum* or when they are force-fed. In addition, weight gain is consistently improved by diethylstilbestrol. The increase in blood lipids

and liver weight occurs independently of the pituitary. Presumably, diethylstilbestrol acts directly on those organs concerned with fat metabolism, such as the liver and adipose tissue and in some way increases the synthesis of lipids from non-lipid precursors. Since the increase in synthesis is not reflected in an increase in the adipose tissues of the hypophysectomized birds, the amount of synthesis induced by estrogen may be considerably less than occurs in intact birds. Lipid synthesis in isolated liver slices is now being studied.

The pituitary in some way participates in the response in intact birds perhaps by maintaining a normal metabolic rate. The caloric requirement for growth and energy production is much higher in the intact birds (Nalbandov and Card, 1942) and the additional caloric demand for increased lipid synthesis is met by increasing food consumption. If the rate of lipid synthesis were very high, a secondary inhibition of the thyroid could occur due to the relative decrease in available energy.

SUMMARY

The effect of diethylstilbestrol on blood and depot fat has been determined in force-fed intact and hypophysectomized cockerels. Hypophysectomized birds tend to have a higher level of blood lipids and depot fat than intact birds. Diethylstilbestrol significantly increases blood lipids in hypophysectomized birds when on a normal or fat-free diet, but has no influence on depot fat. Blood lipids are elevated and depot fat increased in intact birds by both a regular and fat-free diet.

The liver lipids are not influenced by diethylstilbestrol in either intact or hypophysectomized birds.

The thyroid glands and testes are markedly decreased in weight by hypophysectomy but the adrenal glands exhibit only a slight decrease in weight. There were no significant changes in weight of thyroids or adrenals as a result of estrogen treatment in either intact or hypophysectomized cockerels.

Food consumption and rate of gain in intact cockerels are significantly improved by administration of diethylstilbestrol. Efficiency of gain in these birds is not influenced by estrogen.

Diethylstilbestrol has no effect on food consumption, rate of gain, or efficiency of gain in hypophysectomized birds.

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REFERENCES

BAUM, G. J. AND R. K. MEYER: Unpublished data.
BIRD, S.: *Endocrinology* **39**: 149. 1946.

BRENEMAN, W. R.: *Endocrinology* **31**: 179. 1942.
BRIGGS, G. M., T. D. LUCKEY, C. A. ELVEHJEM AND E. B. HART: *J. Biol. Chem.* **148**: 163. 1943.
CLAVERT, J.: *Comp. Rend. soc. Biol.* **138**: 928. 1944.
COMMON, R. H., W. BOLTON AND W. A. RUTLEDGE: *J. Endo.* **5**: 263. 1948.
COMMON, R. H., T. J. KEEFE AND W. A. MAW: *Canadian J. Res.* **28**: D: 272. 1950.
EILERT, M. L.: *Metabolism* **2**: 137. 1953.
ENTENMAN, C., F. W. LORENZ AND I. L. CHAIKOFF: *J. Biol. Chem.* **126**: 133. 1938.
ENTENMAN, C., F. W. LORENZ AND I. L. CHAIKOFF: *J. Biol. Chem.* **134**: 495. 1940.
EPSTEIN, D. I. AND L. F. WOLTERINK: Abstracts of papers presented at the 38th Annual meeting of the Poultry Science Association, August, 1949.
FLEISHMANN, W. AND I. A. FRIED: *Endocrinology* **36**: 406. 1945.
HERTZ, R., J. A. SCHRICKER AND W. W. TULLNER: *Endocrinology* **49**: 168. 1951.
HORLOCK, L. AND L. M. KATZ: *J. Lab. & Clin. Med.* **33**: 733. 1948.
KAR, A. B.: *Anat. Rec.* **97**: 551. 1947.
KUMARAN, J. D. S. AND C. W. TURNER: *Poultry Sci.* **28**: 593. 1949.
LORENZ, F. W.: *Poultry Sci.* **22**: 190. 1943.
LORENZ, F. W., I. L. CHAIKOFF AND C. ENTENMAN: *J. Biol. Chem.* **126**: 763. 1939.
MILLER, R. A. AND O. RIDDLE: *Proc. Soc. Exp. Biol. & Med.* **41**: 518. 1939.
NALBANDOV, A. AND L. E. CARD: *Proc. Soc. Exp. Biol. & Med.* **51**: 294. 1942.
NALBANDOV, A. V. AND L. E. CARD: *J. Exp. Zool.* **94**: 387. 1943.
RANNEY, R. E. AND I. L. CHAIKOFF: *Am. J. Physiol.* **165**: 600. 1951.
RIDDLE, O.: *Endocrinology* **31**: 498. 1942.
RIDDLE, O. AND F. H. BURNS: *Am. J. Physiol.* **81**: 711. 1927.
RIDDLE, O. AND T. SENUM: *Anat. Rec.* (Suppl.), **75**: 58. 1939.
RUSSEL, W. C., M. W. TAYLOR AND L. J. POLSKIN: 1940. *J. Nutrition* **19**: 555. 1940.
STAMLER, J., C. BOLENE, M. DUDLEY AND E. LEVINSON: *Endocrinology* **46**: 375. 1950.
TAUROG, A., F. W. LORENZ, C. ENTENMAN AND I. L. CHAIKOFF: *Endocrinology* **35**: 483. 1944.
THAYER, R. H., R. G. JAAP AND R. PENQUITE: *Poultry Sci.* **23**: 555. 1944.
VAN HORN, L. M.: Personal communication.
ZONDEK, B.: Genital Functions and Their Hormonal Regulation, The Williams and Wilkins Company, Baltimore, Md. Pp. 58–72. 1941.

THE EFFECT OF n-METHYL-PHENYL-ETHYL BARBITURIC ACID ON THYROID FUNCTION IN THE RAT[1]

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INTRODUCTION

THE barbituric acids have been assumed to have no effect, or a slight depressing effect on the thyroidal accumulation of I^{131} (Wase and Greenspan, 1953; Wase *et al.*, 1953). Thus it was somewhat surprising when n-methyl-phenyl-ethyl barbituric acid (MPEBA) was reported to increase the thyroidal accumulation of I^{131} in the rat (Schwarz, 1955). It is the purpose of the present report to present data that confirm and extend the observation that MPEBA enchances the thyroidal accumulation of I^{131} in the rat.

MATERIALS AND METHODS

Ninety-five Sprague-Dawley male rats were maintained on ground commercial rat chow[2] and tap water *ad lib.* from the 23rd day of age until sacrifice at 35 days of age in a temperature controlled animal room (72° F ± 2°). Forty-five of the rats received MPEBA[3] added to the diet at a concentration of 0.1%. Each rat received 2 μc. of carrier free I^{131} [4] by the interperitoneal route and 9 animals from each group were sacrificed at 1.5, 3, 6, 12, and 24 hours after receiving the isotope. The thyroids were removed, weighed, placed in 2 cc. of Bouin's fluid and assayed for I^{131} content in a well-type scintillation counter against three standards of the I^{131} solution as prepared for injection. Radioactivity of the glands was expressed as percent of the administered I^{131}.

Forty-two Sprague-Dawley male rats that had been hypophysectomized[5] on the 21st day of age, and eight intact rats of the same age, were placed on ground commercial rat chow and tap water *ad lib.* in the temperature controlled room on the 28th day of age and were sacrificed on the 36th day of age. Twenty-one of the hypophysectomized rats received MPEBA in the diet at a concentration of 0.1% from the 28th day of age until sacrificed. Nine of the hypophysectomized rats that received the drug also received 1 mg. of thyrotropin[6] per day by subcutaneous injection during the same time interval and

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[2] Purina Laboratory Chow. Ralston Purina Company, St. Louis, Mo.

[3] Mephobarbital (n-methyl-phenyl-ethyl barbituric acid) contributed by Winthrop-Stearns, Inc., New York, N. Y.

[4] I^{131} obtained from Oak Ridge National Laboratory.

[5] Purchased from Hormone Assay Laboratory, Chicago, Ill.

[6] Thyrotropin (No. 317–147) contributed by the Armour Laboratories, Chicago, Ill.

twelve hypophysectomized rats that received no MPEBA treatment were given 1 mg of thyrotropin daily for a similar time interval. All rats received 2 μc. of I^{131} 24 hours before sacrifice and the thyroids were assayed for I^{131} as described above.

Three 50-day old rats that had been maintained on ground commercial rat chow in the temperature controlled room and water *ad lib.* for 27 days and three rats that received MPEBA added to the diet at a concentration of 0.1% from the 23rd to the 45th day of age and then raised to 0.2% until sacrifice, were used for a study of the thyroid clearance of blood iodide. All rats were given 20 μc. of I^{131} by a subcutaneous injection. Ten–25 mg. of blood was collected from the tail in previously weighed capillary hematocrit tubes at 0.5, 1.0, 2, 5, 10, 20, 40, and 60 minutes following isotope administration. The tubes were weighed and assayed for I^{131} as described above and the concentration of I^{131} per gm. of blood calculated. The thyroid gland was removed at 60 minutes post-isotope administration and also assayed for I^{131} content. The thyroid clearance of blood iodide was computed in the usual manner and results expressed as grams of blood cleared per hour by the thyroid gland.

Thirty Sprague-Dawley male rats were maintained on ground commercial rat chow and tap water in the temperature controlled room from the 22nd day of age until sacrifice on the 28th day of age. Fifteen of these rats received MPEBA added to the diet at a concentration of 0.2%. Twenty-nine rats of the same age were handled as above except that the diet was iodine poor,[7] and fifteen of these rats received the drug as above. All of the rats were used for a thyroid:serum I^{131} ratio (T/S) study. Each rat received 6 mg. of a propylthiouracil suspension by subcutaneous injection. One hour later they received 10 μc. of carrier free I^{131} and one hour to one and one-half hours later they were sacrificed. The I^{131} content of the thyroid gland and 0.25 ml. of serum was determined as above. The results were expressed as a ratio of the percent of administered I^{131} found per gram of thyroid to the percent of administered I^{131} found per ml. of serum.

RESULTS

The administration of MPEBA (0.1% of diet for 12 days) enhanced the thyroidal accumulation of I^{131} at 6, 12, and 24 hours after I^{131} administration (Table 1).

TABLE 1. 35-DAY OLD SPRAGUE-DAWLEY MALE RATS MAINTAINED ON CONTROL DIET OR CONTROL DIET PLUS n-METHYL-PHENYL-ETHYL BARBITURIC ACID (0.1%) FROM 23RD DAY OF AGE. MEAN VALUE PLUS OR MINUS STANDARD ERROR. WHEN MEAN IS ITALICIZED, IT IS DIFFERENT FROM MEAN IMMEDIATELY ABOVE, BY t-TEST, $P = 0.01$

Treatment	No.	Hrs. between I^{131} and sacrifice	% of administered I^{131} in thyroid gland	% of administered I^{131} per mg. of thyroid
Control	9	1.5	2.1 ± 0.3	0.23 ± 0.04
MPEBA	9	1.5	1.8 ± 0.1	0.22 ± 0.01
Control	9	3.0	4.0 ± 0.3	0.41 ± 0.01
MPEBA	9	3.0	5.3 ± 0.5	0.50 ± 0.05
Control	9	6.0	5.7 ± 0.4	0.55 ± 0.05
MPEBA	9	6.0	*8.0* ± 0.5	*0.80* ± 0.06
Control	9	12.0	7.9 ± 0.8	0.86 ± 0.13
MPEBA	9	12.0	*13.3* ± 0.8	*1.38* ± 0.06
Control	9	24.0	9.3 ± 0.2	1.08 ± 0.05
MPEBA	9	24.0	*13.6* ± 1.0	*1.35* ± 0.01

[7] Remington Diet No. 348. Each 100 gm. contains: Wheat gluten 16 gm., dried yeast 4 gm., corn meal 78 gm., $CaCO_3$ 1 gm., and NaCl 1 gm.

TABLE 2. 36-DAY OLD SPRAGUE-DAWLEY MALE RATS. HYPOPHYSECTOMIZED ON 21ST DAY. MAINTAINED ON CONTROL DIET OR CONTROL DIET PLUS D-METHYL-PHENYL-ETHYL BARBITURIC ACID (0.1%) FROM 28TH DAY UNTIL SACRIFICE. THYROTROPIN (1 MG. PER DAY) FROM 28TH DAY UNTIL SACRIFICE. MEAN VALUE PLUS OR MINUS STANDARD ERROR. WHEN MEAN IS ITALICIZED, IT IS DIFFERENT FROM MEAN IMMEDIATELY ABOVE, BY t-TEST, P = 0.01

Treatment	No.	Body weight (gm.)	Thyroid weight (mg.)	% administered I^{131} in thyroid gland
Intact	8	133 ±3	8.5 ±0.7	9.6 ±0.4
Hypophysectomized	11	*60* ±2	*3.3* ±0.2	*0.5* ±0.1
Hypox + MPEBA	12	60 ±2	3.9 ±1.2	0.4 ±0.1
Hypox + TSH	10	66 ±6	*5.7* ±0.4	*11.9* ±0.9
Hypox + MPEBA + TSH	9	67 ±2	5.6 ±0.5	*15.2*[1] ±1.2

[1] P + 0.05.

It was evident that the drug (0.1% of diet for 7 days) produced no enhanced accumulation of I^{131} in hypophysectomized rats unless exogenous thyrotropin (1 mg. per day for 7 days) was supplied (Table 2).

When the thyroid-serum iodide ratio was studied, MPEBA (0.2% of diet for 5 days) increased the ratio in the adequate iodine diet and low iodine diet animals (Table 3).

TABLE 3. 28-DAY OLD SPRAGUE-DAWLEY MALE RATS. MAINTAINED ON CONTROL DIET OR CONTROL DIET PLUS D-METHYL-PHENYL-ETHYL BARBITURIC ACID (0.2%) FROM 22ND DAY UNTIL SACRIFICE OR ON LOW-IODINE DIET OR LOW-IODINE DIET PLUS DRUG IN SAME CONCENTRATION, FOR SAME PERIOD. MEAN PLUS OR MINUS STANDARD ERROR. WHEN MEAN IS ITALICIZED, IT IS DIFFERENT FROM MEAN IMMEDIATELY ABOVE, BY t-TEST, P = 0.01

Treatment	No.	$T/S = \frac{\%\text{ administered } I^{131}/\text{gm. thyroid}}{\%\text{ administered } I^{131}/\text{cc. serum}}$
Control	15	28.2 ±1.1
Control + MPEBA	14	*48.6* ±2.3
Low Iodine	15	*122.3* ±6.4
Low Iodine + MPEBA	15	*154.2* ±9.9

The clearance of I^{131} from the blood by the thyroid gland was elevated when MPEBA was fed (0.1% for 22 days and 0.2% for last 5 days) (Table 4).

The administration of the drug had no consistent effect on body weight nor any untoward effects other than a slight drowsiness that persisted through the first 3–4 days of drug administration.

TABLE 4. 50-DAY OLD SPRAGUE-DAWLEY MALE RATS, MAINTAINED ON CONTROL DIET FROM 35TH DAY OF AGE UNTIL SACRIFICE OR CONTROL DIET PLUS D-METHYL-PHENYL-ETHYL BARBITURIC ACID (DAYS 34–45, 0.1%; DAY 45, 0.2% UNTIL SACRIFICE). MEAN VALUE PLUS OR MINUS STANDARD ERROR. WHEN MEAN IS ITALICIZED, IT IS DIFFERENT FROM CONTROL MEAN, BY t-TEST, P = 0.01

Treatment	No.	Gm. blood cleared per hr. by thyroid
Control	3	2.0 ±0.5
MPEBA	3	*4.1* ±0.2

DISCUSSION

It seems clearly established, from the report of Schwarz (1955) and the data here reported, that n-methyl-phenyl-ethyl-barbituric acid enhances the capacity of the rat thyroid gland to accumulate I^{131}. Thus, the effects of this compound on thyroid function are not similar to those of sodium 5-ethyl-5-(1 methylbutyl) barbiturate or sodium 5-ethyl-5-(1 methyl, 1 butenyl) barbiturate which have no effect on the accumulation of I^{131} by the rat thyroid gland (Wase *et al.*, 1953), or sodium 5-ethyl-5-(1 methylbutyl) thiobarbiturate and sodium 5-allyl-5-(1 methylbutyl) 2-thiobarbiturate which depress the accumulation of I^{131} by the rat thyroid gland (Wase and Greenspan, 1953, Wase, *et al.*, 1953), but closely resemble the action of 5,5-diethylbarbituric acid and 5-ethyl-5-phenylbarbituric acid which also increase the accumulation of I^{131} by the rat thyroid gland (Schwarz, *et al.*, 1955).

The fact that the thyroid gland cleared I^{131} from the blood at a faster rate when the animals were given MPEBA indicates that, in the present study, the action of the compound was not due to a depressing effect on renal clearance of I^{131} which would have in turn presented more I^{131} to the thyroid gland but rather that MPEBA acts directly on the thyroid-pituitary axis. It has been demonstrated in the present study, and by similar experiments reported by Schwarz *et al.* (1955), that MPEBA was not effective in the hypophysectomized rat unless exogenous thyrotropin was given. It would thus appear that the compound can act at the level of the thyroid gland. However, these experiments with hypophysectomized animals do not exclude the possibility that the compound might act at the level of the pituitary gland in the intact animal.

The mechanism by which MPEBA might act on the thyroid gland to produce an enhanced collection of I^{131} is not clear. In the present experiment, the compound increased the iodide trap which suggests that there was an increased rate of entry of iodide into the thyroid gland. Schwarz *et al.* (1955) believes that the compound stimulates the entire iodine metabolism of the thyroid gland and suggests that thyroid glands from rats treated with MPEBA produced an iodine compound different from ordinary thyroid hormone. It seems reasonable to conclude, from the present study and the reports of Schwarz, the MPEBA can act to enhance the thyroidal accumulation of I^{131} by a direct action upon the thyroid gland.

SUMMARY

N-methyl-phenyl-ethyl barbituric acid, when fed to young Sprague-Dawley male rats, enhanced the thyroidal accumulation of I^{131}, increased the clearance of I^{131} from the blood by the thyroid gland, and increased the thyroid-serum iodide ratio. The compound was not effective in hypophysectomized rats in the absence of exogenous thyrotropin but thyro-

tropin restored the response. It was suggested that the compound acts directly on the thyroid gland to enhance the accumulation of I^{131} but the mechanism of this action was not clear.

REFERENCES

REMINGTON, R. E.: *J. Nutrition* **13**: 223. 1937.
SCHWARZ, F.: *Acta Endocrinol.* **18**: 33. 1955.
SCHWARZ, F., P. J. KINDEREN, J. L. VAN HAAFTEN AND L. E. KOOPMAN: *Acta Endocrinol.* **19**: 411. 1955.
WASE, A. W. AND J. GREENSPAN: *Proc. Soc. Exper. Biol. and Med.* **84**: 154. 1953.
WASE, A. W., E. REPPLINGER AND W. C. FOSTER: *Endocrinology* **53**: 630. 1953.

ENDOCRINE INFLUENCES UPON ESTERASE ACTIVITY OF RAT BONE MARROW[1]

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IT IS now established that endocrine factors exert a regulatory influence on the numbers and types of cells within the circulating blood and blood-forming organs (Gordon and Charipper, 1947; Daughaday, Williams, and Daland, 1948; Gordon, 1954, 1955). Recently, attention has turned to the alterations induced in the metabolism of bone marrow and leucocytes by a variety of factors, including those of endocrine origin (Beck and Valentine, 1953; Gordon, 1954, 1955; Martin, McKinney, and Green, 1955). In this connection, Evans and Bird (1949) have suggested that fat plays an important role in normal rabbit bone marrow and have demonstrated that marrow has the ability to utilize fat in the absence of carbohydrate. Evans (1952) has shown recently that rabbit bone marrow displays a high degree of esterase activity when compared to other tissues of the same animal. It has been found that polymorphonuclear leucocytes, obtained from peritoneal exudates, contain an enzyme capable of hydrolyzing tributyrin and methyl butyrate (Rossiter and Wohg, 1949). Since esterase activity appears to represent a responsive parameter of marrow, it was of interest to study the effects of some endocrine deficiencies and replacement treatment upon this enzyme system.

METHODS

Adult male rats of a modified Long-Evans strain, ranging in weight from 190–215 gm. were employed. They were kept on a standard laboratory ration and fed ad libitum. All adrenalectomized animals received 1% saline as the drinking fluid. All hypophysectomized animals were used experimentally 6–8 weeks following pituitary removal.

The experimental groups consisted of: (1) normal intact rats; (2) adrenalectomized animals sacrificed at 5 days and at 2 weeks following the operation; (3) adrenalectomized animals received cortisol (17-OH corticosterone); (4) hypophysectomized animals; and (5) hypophysectomized animals treated with growth hormone (GH).

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[2] Taken from a thesis presented by Herbert Megel to the Faculty of the Graduate School of Arts and Science, New York University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Biology.

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The adrenalectomized rats receiving cortisol (free alcohol)[4] were injected subcutaneously for a period of 7 days with 3 mg. of this steroid suspended in 1 ml. of physiological saline, 2 daily injections being given prior to the operation, and 5 daily injections after adrenal removal. Control animals for this group consisted of adrenalectomized rats receiving injections of only saline. All animals were sacrificed upon the 8th day.

The hypophysectomized animals treated with GH were given subcutaneously, 1 mg. of a Raben-Westermeyer preparation[5] solubilized in 0.2 ml. of acidified water (pH 3.0) daily for a period of 7 days. The control animals were hypophysectomized rats receiving 0.2 ml. of acidified water for the same length of time. These animals were also sacrificed upon the 8th day.

Esterase activity was determined manometrically by the method of Gordon (1952). Tributyrin and methyl butyrate served as substrates. Rat bone marrow was homogenized in cold glass-redistilled water and placed into an ice bath until used. Five-tenths ml. of marrow homogenate was added to each flask followed by 0.5 ml. of a distilled water-bicarbonate solution to yield a final concentration of bicarbonate equivalent to 0.027 M. Two-tenths ml. of substrate was then added to each reaction vessel. The reaction mixture was gassed for a 15 minute period with a 5% carbon dioxide and 95% nitrogen mixture to provide an anaerobic atmosphere, and to adjust the pH to 7.6. After gassing, the flasks were equilibrated against atmospheric pressure for 10 minutes. Four 10 minute readings were taken. The first 10 minute reading was discarded, allowing for an additional equilibration period. The results are expressed as microliters of carbon dioxide evolved per 30 min. per mg. of total nitrogen—nitrogen determined by the method of Miller and Miller (1939). In some instances, the results are based upon 10^8 nucleated marrow cells, these counts having been determined from the marrow suspensions by standard hemacytometer methods.

One flask, not containing substrate, served as a control for any endogenous acid production from cells not homogenized. All gas evolution from flasks to which substrate was added, was corrected for endogenous acid production. Since tributyrin and methyl butyrate showed no spontaneous hydrolysis, control vessels for the substrate esters were eliminated.

Esterase activity, using these same substrates, was also determined upon marrow cell suspensions. The experimental groups were the same as those described for the homogenate study. The manometric procedure was identical with that indicated for the homogenate, except that the cells were suspended in isotonic Krebs-Ringer solution. The effect of Krebs-Ringer solution upon bone marrow homogenates of normal rats was also studied.

RESULTS

Esterase activity of rat bone marrow homogenates, as measured by tributyrin and methyl butyrate hydrolysis, was not altered by adrenalectomy, but was elevated significantly following hypophysectomy (Table 1). This same pattern was revealed when marrow suspensions were used instead of homogenates. In all cases, the esterase activity of marrow suspensions was significantly greater than that of homogenates from identical experimental groups (Table 1).

Cortisol administered to the adrenalectomized rats failed to modify ester hydrolysis of rat bone marrow suspensions when the results were

[4,5] We thank Dr. H. J. Robinson, Merck and Co., Inc., for providing the cortisol and Dr. W. Kleinberg, Princeton Laboratories, Inc., for the GH.

TABLE 1. EFFECTS OF ENDOCRINE DEFICIENCIES UPON ESTERASE ACTIVITY OF RAT BONE MARROW HOMOGENATES AND SUSPENSIONS

μl. CO_2/mg. N./30 min.
(Means ± Standard Errors)

Substrates	Normal-Control	Adrenalectomy 2 weeks	Adrenalectomy 5 days	Hypophysectomy 6–8 weeks
		HOMOGENATES		
Tributyrin	74.5 ± 2.47 (7)	76.0 ± 4.44 (8)	67.8 ± 2.47 (10)	114.4 ± 12.6 (5) P(0.004)
Methyl Butyrate	6.4 ± 1.07 (7)	7.1 ± 1.83 (7)	4.9 ± 0.75 (10)	25.5 ± 3.75 (5) P(0.001)
		SUSPENSIONS		
Tributyrin	95.6 ± 2.56 (17)	—	99.4 ± 3.63 (8)	138.0 ± 14.6 (8) P(0.001)
Methyl Butyrate	16.8 ± 1.92 (4)	—	14.0 ± 1.43 (8)	48.4 ± 4.60 (8) P(0.001)

( ) Denotes number of animals in each experimental group.
P( ) Probability values obtained from the distribution of Fisher's t.

related to the total nitrogen values (Table 2). However, a marked elevation of tributyrin hydrolysis was observed when the same carbon dioxide evolution was calculated upon the basis of cellular numbers (Table 2). The increase in hydrolysis of methyl butyrate was not as significant as that for tributyrin.

The bone marrow of the hypophysectomized animal, already displaying a heightened enzyme activity compared to the normal controls, showed an even greater capacity for splitting tributyrin following GH therapy.

TABLE 2. EFFECTS OF CORTISOL UPON ESTERASE ACTIVITY OF CELL SUSPENSIONS OF MARROW FROM ADRENALECTOMIZED RATS

(Means ± Standard Errors)

Substrates	μl. CO_2/mg. N./30 min.		μl. CO_2/10^8 cells/30 min.	
	Adrenalectomy plus saline[1] control	Adrenalectomy plus cortisol[2]	Adrenalectomy plus saline[1] control	Adrenalectomy plus cortisol[2]
Tributyrin	111.1 ± 3.3 (6)	116.4 ± 7.3 (6)	201 ± 7.3 (6)	257 ± 16.6 (6) P(0.010)
Methyl Butyrate	24.1 ± 5.7 (6)	38.7 ± 8.7 (6)	43.7 ± 9.9 (6)	84.0 ± 20.3 (6) P(trend)

( ) Denotes numbers of rats in each experimental group.
P( ) Probability values obtained from the distribution of Fisher's t.
[1] These rats were given 1.0 ml. of saline per day for a period of 7 days, 2 days prior to adrenalectomy and for 5 days following the operation.
[2] These rats received 3.0 mg. of cortisol suspended in 1.0 ml. of saline per day for a period of 7 days, 2 days prior to adrenalectomy and for 5 days following the operation. All animals were sacrificed upon the eighth day.

TABLE 3. EFFECTS OF GH UPON ESTERASE ACTIVITY OF CELL SUSPENSIONS OF MARROW FROM HYPOPHYSECTOMIZED RATS

(Means ± Standard Errors)

Substrates	μl. CO_2/mg. N./30 min.		μl. CO_2/10^8 cells/30 min.	
	Hypophysectomy plus acidified water[1]	Hypophysectomy plus GH[2]	Hypophysectomy plus acidified water[1]	Hypophysectomy plus GH[2]
Tributyrin	129.8 ± 6.7 (10)	153.2 ± 7.3 (12) P(0.05)	242 ± 7.6 (10)	264 ± 11.2 (12) P(0.07)
Methyl Butyrate	33.2 ± 3.2 (10)	33.0 ± 4.7 (9)	56.3 ± 6.1 (10)	53.8 ± 6.2 (9)

( ) Denotes numbers of animals in each experimental group.
P( ) Probability values obtained from the distribution of Fisher's t.
[1] These hypophysectomized animals received 0.2 ml. of acidified water (pH 3.0) per day for a period of 7 days.
[2] These hypophysectomized animals received 1.0 mg. of growth hormone in 0.2 ml. acidified water per day for a period of 7 days. All animals were sacrificed upon the eighth day.

This hormonal treatment was not effective in modifying methyl butyrate hydrolysis (Table 3). These same results were obtained whether total nitrogen or cellular numbers served as a basis for activity comparison (Table 3).

The enzyme activity of the marrow suspensions was significantly greater than that for the homogenates in similarly treated experimental groups. In order to test whether the incubation medium could have caused this discrepancy, bone marrow homogenates were incubated in a Krebs-Ringer bicarbonate solution to which tributyrin was added. Similar aliquots of the marrow homogenate were incubated in a water-bicarbonate tributyrin mixture. These studies were repeated using methyl butyrate as substrate. The results (Table 4) indicate that Krebs-Ringer medium actually inhibited ester hydrolysis of homogenates, and therefore could not be responsible for the increased activity of the marrow suspension.

DISCUSSION

It seems clear from the data that certain endocrine modifications alter the esterase enzyme of rat bone marrow. Adrenalectomy is without ap-

TABLE 4. INHIBITION OF ESTERASE ACTIVITY OF NORMAL RAT BONE MARROW HOMOGENATES WITH KREBS-RINGER SOLUTION

μl. CO_2/mg. N./30 min.

Substrates	Water and bicarbonate	Krebs-Ringer and bicarbonate	Absolute change ± standard errors	% inhibition	Probability values
Tributyrin	78.2 (5 animals)	62.4	−15.8 ± 1.28	20.3	0.001
Methyl Butyrate	14.1 (5 animals)	10.2	− 3.9 ± 0.88	27.9	0.008

parent effect while cortisol treatment seems to elevate ester hydrolysis only when the results are based upon cellular numbers. This suggests that the corticoid may have altered the nitrogen content per cell rather than have affected the enzymatic activity *per se*.

Hypophysectomy results in an elevation in the esterase activity of rat bone marrow compared to the normal controls. GH administered to the hypophysectomized animals, produces an even greater esterase activity of marrow towards tributyrin. It is ineffective, however, in altering the hydrolysis of methyl butyrate. This relationship is observed when either total nitrogen or cellular numbers are used as bases. It would appear likely that more than one enzyme affecting ester hydrolysis exists in marrow, since the rates at which both substrates are hydrolyzed are not increased proportionally with similar treatment.

Chemical analyses have shown that lipid comprises a relatively high percentage of marrow (McCay and Schultze, 1944). This lipid moiety is extremely labile and can be depleted easily by hemorrhage (Evans and Oppenheimer, 1955), phenylhydrazine treatment (Krause, 1943; Evans, Baker and Oppenheimer, 1955), or benzene poisoning (Dietz and Steinberg, 1950). Marrow fat can also be altered through endocrine procedures. Thus, thyroidectomy (Gordon, Kadow, Finkelstein and Charipper, 1946) and hypophysectomy (Vollmer, Gordon, Levenstein and Charipper, 1939) cause infiltration of fat within the marrow. Fruhman, Gerstner and Gordon (1954) observed that the hypoplastic marrow, produced following hypophysectomy, can be repaired considerably with GH. Following GH treatment, a decrease in marrow fat and an increase in marrow cellularity was observed. Further studies relating the alterations in esterase activity to the metabolism of fat and changes in cellular numbers and types within the blood forming organs should serve to illuminate more fully the signifiance of lipins in hemopoietic processes.

A significant difference in ester hydrolysis was encountered in comparing the enzyme activities of marrow suspensions to marrow homogenates from similarly treated experimental groups. In all cases, the esterase activity of marrow suspensions was significantly greater than the homogenates. This phenomenon is not due to the difference in incubation media since the homogenate activity tested in the medium used for suspensions (Krebs-Ringer solution) inhibited, rather than accelerated ester hydrolysis.

It can also be considered that this difference in enzyme activity may depend upon cellular integrity as compared to the broken cell. With the homogenate, the end products are those of hydrolysis. With the suspension, not only are the end products of hydrolysis produced, but some of these may also be metabolized further to acidic substances that cannot be differentiated manometrically from the acids produced through hydrolysis. Thus the increased activity observed with the cellular suspension might be

due to a hydrolytic product of tributyrin, such as the glycerol moiety, which can enter into the metabolic pattern of carbohydrate utilization and give rise to an acidic substance. An attempt was made to analyze chemically the incubating solutions for other acids, such as lactic acid (method of Barker and Summerson, 1939) which could arise from glycerol. It was found, however, that glycerol and tributyrin interfered with and gave a colorimetric reaction similar to that for lactic acid. Therefore, direct evidence to support this concept is still needed.

A similar explanation may hold for the increase in methyl butyrate hydrolysis by the suspension. With homogenates, methanol and butyric acid are formed upon hydrolysis. Both these products are formed with the suspension, but the methanol may be oxidized further to formic acid. Thus for every mole of methyl butyrate completely hydrolyzed, one mole of acid is produced by the homogenate and two moles of acid may be formed in the suspension. The data (Table 1) offer quantitative support for this concept.

SUMMARY

Hypophysectomy in the rat results in an elevation of bone marrow esterase activity (total nitrogen basis) towards the substrates tributyrin and methyl butyrate, when either marrow homogenates or suspensions are employed. Esterase activity of bone marrow suspensions towards tributyrin is further increased following administration of GH to hypophysectomized animals; this holds whether the results are expressed on a total nitrogen or nucleated cell number basis. Esterase activity is unaffected by adrenalectomy, but is increased following treatment with cortisol when activity is based upon numbers of nucleated cells.

Bone marrow cell suspensions display greater esterase activity than do homogenates. This may be due to the ability of the intact cell to metabolize further the hydrolytic products.

REFERENCES

BARKER, S. B. AND W. H. SUMMERSON: *J. Biol. Chem.* **138**: 535. 1941.
BECK, W. S. AND W. N. VALENTINE: *Cancer Research* **13**: 309. 1953.
DAUGHADAY, W. H., R. H. WILLIAMS AND G. A. DALAND: *Blood* **3**: 1342. 1948.
DIETZ, A. A. AND B. STEINBERG: *Arch. Biochem.* **26**: 291. 1950.
EVANS, J. D.: *Am. J. Physiol.* **171**: 48. 1952.
EVANS, J. D. AND R. M. BIRD: *J. Biol. Chem.* **181**: 357. 1949.
EVANS, J. D. AND M. J. OPPENHEIMER: *Am. J. Physiol.* **181**: 509. 1955.
EVANS, J. D., J. M. BAKER AND M. J. OPPENHEIMER: *Am. J. Physiol.* **181**: 504. 1955.
FRUHMAN, G., R. GERSTNER AND A. S. GORDON: *Proc. Soc. Exp. Biol. & Med.* **85**: 93. 1954.
GORDON, A. S.: *Recent Progress in Hormone Research.* Academic Press. New York, N. Y. **10**: 339. 1954.
GORDON, A. S.: *Ann. N. Y. Acad. Sci.* **59**: 907. 1955.

GORDON, A. S. AND H. A. CHARIPPER: *Ann. N. Y. Acad. Sci.* **48**: 615. 1947.
GORDON, A. S., P. C. KADOW, G. FINKELSTEIN AND H. A. CHARIPPER: *Am. J. Med. Sci.* **212**: 385. 1946.
GORDON, J. J.: *Biochem. J.* **51**: 97. 1952.
KRAUSE, R. F.: *J. Biol. Chem.* **149**: 395. 1943.
MARTIN, S. P., G. R. MCKINNEY AND R. GREEN: *Ann. N. Y. Acad. Sci.* **59**: 996. 1955.
MCCAY, R. H. AND M. O. SCHULTZE: *J. Biol. Chem.* **156**: 479. 1944.
MILLER, G. L. AND E. E. MILLER: *Anal. Chem.* **20**: 481. 1948.
ROSSITER, R. J. AND E. WONG: *J. Biol. Chem.* **180**: 993. 1949.
VOLLMER, E. P., A. S. GORDON, I. LEVENSTEIN AND H. A. CHARIPPER: *Endocrinology* **25**: 970. 1939.

EFFECT OF LYOPHILIZED PLASMA AND PLASMA FRACTIONS FROM HYPOPHYSEAL-PORTAL VESSEL BLOOD ON ADRENAL ASCORBIC ACID[1]

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THE resolution of factors influencing the release of the adrenocorticotrophic hormone (ACTH) is a problem of continuing interest among investigators. It is noteworthy that destructive lesions in the hypothalamus (De Groot and Harris, 1950; Hume and Wittenstein, 1950; Porter, 1953; McCann, 1953; McCann and Sydnor, 1954; Ganong and Hume, 1954; and Laqueur *et al.*, 1955), pituitary transplants (Harris and Jacobsohn, 1952 and Jacobsohn, 1954), and tissue cultures of the anterior lobe (Guillemin, 1955) suggest that the hypothalamus is of significance in regulating ACTH secretion, possibly by means of a neurohumoral substance (Hinsey and Markee, 1933 and Green and Harris, 1947). The vascular requirements for the direct transport of such a blood-borne substance from the hypothalamus to the anterior lobe are present as portal vessels (Wislocki and King, 1936; Green and Harris, 1947, 1949; and Xuereb *et al.*, 1954). It has been observed that plasma from the hypothalamico-hypophyseal portal vessels promotes the release of ACTH from the anterior lobe of the pituitary as measured by the adrenal ascorbic acid depletion test (Porter and Jones, 1956).

This investigation deals with the effect of lyophilized plasma and plasma protein fractions from hypophyseal portal vessel blood as well as Pitressin on adrenal ascorbic acid of the intact rat.

MATERIALS AND METHODS

The plasma used in these experiments was obtained from blood of 19 mongrel dogs of both sexes varying in weight from 9.6 to 26 kg. Blood for experimental purposes was obtained from the hypothalamico-hypophyseal portal vessels; whereas, control blood was obtained from the common carotid artery. The procedure employed was the same as that reported by Porter and Jones (1956). The plasma from each blood sample was removed following centrifugation and was stored in the frozen state if not used immediately.

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Lyophilized plasma for experimental purposes was obtained from 266 ml. of portal plasma. The lyophilized residues were pooled, mixed to insure homogeneity, and weighed. In removing aliquots for assay 0.0795 gm. was considered to be equivalent to 1 ml. of portal vessel plasma. Control plasma from the carotid artery was treated in the same manner. All lyophilized residues were kept in a desiccator at 4° C.

For purification 86 ml. of pooled portal vessel plasma was dialyzed[2] with stirring for 45 hours at 4° C against a total volume of 12 liters of 0.9% NaCl solution. The dialyzed plasma was then filtered through Whatman #42 filter paper, and an aliquot of the filtrate equivalent to 20 ml. of the original plasma was lyophilized and used for assay in the ascorbic acid depletion test. The remaining 66 ml. as well as 96 ml. of non-dialyzed plasma were fractionated separately according to the method of Cohn *et al.* (1946). Each precipitate was next dissolved in isotonic saline and dialyzed for 2 hours at 4° C against 1 liter of 0.9% saline and then diluted with isotonic saline to one-half the plasma volume used for fractionation. The supernatant was dialyzed against saline without dilution. Aliquots from all fractions were lyophilized for assay. The remainder was stored at −10° C.

For the determination of electrophoretic mobility 5 ml. of precipitate II+III[3] was dialyzed at 4° C against 2 liters of veronal buffer (pH 8.6; ionic strength 0.1). The sample was then run in a 2 ml. cell of a Perkin-Elmer Model 38 electrophoresis apparatus.

The effect of the lyophilized whole plasma and plasma protein fractions from portal blood, and of Pitressin (Parke, Davis and Co.) on adrenal ascorbic acid was determined using adult, male, albino rats of the Sprague-Dawley strain. The types of animal preparations employed were (1) the hypophysectomized rat and (2) the hydrocortisone-inhibited, intact rat. The hypophysectomized rats were prepared by the parapharyngeal approach and were used in the adrenal ascorbic acid depletion test 20 to 24 hours following hypophysectomy (Sayers, Sayers, and Woodbury, 1948). Gross examination of the sella area was performed at autopsy and results from all animals showing incomplete operations were discarded. Hydrocortisone-inhibited, intact rats were prepared by injecting intraperitoneally 6 mg. of hydrocortisone acetate in saline per 100 gm. body weight 4 hours before use in the adrenal ascorbic acid depletion test.

All solutions were administered via the tail vein in two injections of 2 ml. per injection. The first injection was given immediately after removal of the left adrenal, and the second was given 30 minutes later. The right adrenal was removed 90 minutes after the first injection. All solutions were prepared just prior to use. The first adrenal was removed under ether anesthesia; the second adrenal was removed under nembutal anesthesia. Lyophilized plasma from the portal vessels and carotid artery as well as the lyophilized portal plasma protein fractions were dissolved in distilled water and the concentration adjusted so that 4 ml. of the solution injected was equivalent to a known volume of whole plasma. Pitressin was diluted to the desired concentration with 0.9% NaCl.

RESULTS

The effects of injections of lyophilized plasma equivalent to 10 ml. of plasma from the portal vessels on adrenal ascorbic acid of hydrocortisone-inhibited, intact rats are presented in Table 1. The ascorbic acid reduction (mg. per 100 gm. adrenal weight) in intact rats receiving portal plasma was -107 ± 12.9; whereas, the change in intact rats receiving carotid artery plasma was 0 ± 2.8 and was -14 ± 5.4 in hypophysectomized rats receiv-

[2] No. 4465-AZ Dialyzer Tubing, A. H. Thomas Co., Philadelphia.

[3] Designations of protein fractions from portal vessel plasma are those used by Cohn *et al.* (1946).

TABLE 1. EFFECT OF LYOPHILIZED PLASMA ON ADRENAL ASCORBIC ACID OF HYDROCORTISONE-INHIBITED, INTACT RATS AND HYPOPHYSECTOMIZED RATS

Number of rats	Weight of rats (gm.)	Initial treatment	Ascorbic acid concentration left adrenal (at 0 time) (mg./100 gm.) (mean)	Final treatment (lyophilyzed blood plasma, i.v.)	Ascorbic acid concentration right adrenal (at 90 min.) (mg./100 gm.) (mean)	Difference in ascorbic acid concentration (left adrenal minus right adrenal) (mg./100 gm.)
9	288±8.1*	6 mg./100 gm. body weight Hydrocortisone Acetate, i.p.	370	*0 min.*—Lyophilized plasma equivalent to 5 ml. of carotid artery plasma *30 min.*—Lyophilized plasma equivalent to 5 ml. of carotid artery plasma	370	0± 2.8
9	298±6.1	6 mg./100 gm. body weight Hydrocortisone Acetate, i.p.	380	*0 min.*—Lyophilized plasma equivalent to 5 ml. of portal vessel plasma *30 min.*—Lyophilized plasma equivalent to 5 ml. of portal vessel plasma	273	−107±12.9**
9	266±3.7	Hypophysectomized 20–24 hours before use	430	*0 min.*—Lyophilized plasma equivalent to 5 ml. of carotid artery plasma *30 min.*—Lyophilized plasma equivalent to 5 ml. of carotid artery plasma	427	− 3± 6.1
10	294±9.3	Hypophysectomized 20–24 hours before use	404	*0 min.*—Lyophilized plasma equivalent to 5 ml. of portal vessel plasma *30 min.*—Lyophilized plasma equivalent to 5 ml. of portal vessel plasma	390	− 14± 5.4

* Mean and Standard Error.
** Differs significantly from controls ($P<0.01$).

ing portal plasma. The reduction observed when intact rats were injected with portal plasma was significantly greater than that observed in intact rats receiving carotid artery plasma or hypophysectomized rats receiving portal plasma ($P<0.01$).

Ascorbic acid reduction in intact rats injected with dialyzed, lyophilized portal plasma equivalent to 10 ml. of plasma was −103 (Table 2). The dialysis was against 0.9% NaCl for 45 hours at 4° C. Following alcohol fractionation of portal plasma (Cohn *et al.*, 1946), the only fraction which caused a decrease in ascorbic acid was precipitate II+III. Injections of precipitate II+III in amounts equivalent to 10 ml. and 22 ml. of portal

TABLE 2. EFFECT OF PROTEIN FRACTIONS OF PORTAL VESSEL PLASMA ON ADRENAL ASCORBIC ACID OF THE HYDROCORTISONE-INHIBITED, INTACT RAT

Number of rats	Ascorbic acid concentration left adrenal (at 0 min.) (mg./100 gm.) (mean)	Fraction injected from portal vessel plasma*—injected at 0 min. and at 30 min.	Total quantity injected (plasma equivalent in ml.)†	Ascorbic acid concentration right adrenal (at 90 min.) (mg./100 gm.) (mean)	Difference in ascorbic acid concentration (left adrenal minus right adrenal) (mg./100 gm.)
2	349	Dialyzed, lyophilized plasma	10	246	−103
3	422	Precipitate II+III	10	306	−116
1	400	Precipitate II+III	22	181	−219
2	362	Precipitate IV-1	10	387	+ 25
1	285	Precipitate IV-1	22	306	+ 21
2	322	Precipitate IV-4	10	348	+ 26
1	302	Precipitate IV-4	22	310	+ 8
2	347	Precipitate V	10	343	− 4
1	317	Precipitate V	22	358	+ 41
2	375	Precipitate I	10	398	+ 23
2	341	Supernatant	10	375	+ 34

* Dissolved in distilled H_2O and injected intravenously in two injections of 2 ml. each.
† The volume indicated is the volume of original portal vessel plasma which the lyophilized fraction or portion used represents.

TABLE 3. EFFECT OF PITRESSIN ON ADRENAL ASCORBIC ACID OF HYDROCORTISONE-INHIBITED, INTACT RATS AND HYPOPHYSECTOMIZED RATS

Number of rats	Weight of rats (gm.)	Initial treatment	Ascorbic acid concentration left adrenal (at 0 time) (mg./100 gm.) (mean)	Final treatment (pitressin, i.v.)	Ascorbic acid concentration right adrenal (at 90 min.) (mg./100 gm.) (mean)	Difference in ascorbic acid concentration (left adrenal minus right adrenal) (mg./100 gm.)
6	283± 4.3*	6 mg./100 gm. body weight hydrocortisone acetate, i.p.	374	*0 min.*—2 ml. pitressin† equivalent to 0.15 units *30 min.*—2 ml. pitressin equivalent to 0.15 units	329	− 45± 3.1
6	313±18.7	6 mg./100 gm. body weight hydrocortisone acetate, i.p.	397	*0 min.*—2 ml. pitressin equivalent to 0.3 units *30 min.*—2 ml. pitressin equivalent to 0.3 units	344	− 53±13.5
6	353± 8.9	6 mg./100 gm. body weight hydrocortisone acetate, i.p.	393	*0 min.*—2 ml. pitressin equivalent to 0.8 units *30 min.*—2 ml. pitressin equivalent to 0.8 units	292	−101± 5.1
6	317±17.1	6 mg./100 gm. body weight hydrocortisone acetate, i.p.	394	*0 min.*—2 ml. pitressin equivalent to 1.2 units *30 min.*—2 ml. pitressin equivalent to 1.2 units	278	−106± 5.2
7	279± 7.3	Hypophysectomized 20–24 hours before use.	385	*0 min.*—2 ml. pitressin equivalent to 0.8 units *30 min.*—2 ml. pitressin equivalent to 0.8 units	349	− 36± 7.7

* Mean and S.E.
† Pitressin—Parke, Davis and Co.

plasma into intact rats caused reductions in ascorbic acid of −116 and −219 respectively (Table 2). Electrophoretic analysis of this fraction revealed the presence of at least four components. These components had the following mobilities[4] and relative percentages: 1.6±0.2 (32%), 2.5±0.15 (36%), 3.8±0.04 (20%), and 6.9±0.15 (7%).

When Pitressin was injected into hydrocortisone-inhibited, intact rats in graded quantities varying from 0.3 to 2.4 units, the mean decrease in ascorbic acid changed from −45±3.1 to −106±5.2 (Table 3). The decrease in hypophysectomized rats receiving 1.6 units of Pitressin was −36±7.7; whereas, the change in intact rats injected with 1.6 units was −101±5.1. This difference is significant ($P < 0.01$). It is of interest that the difference in response to 1.6 units and to 2.4 units was not significant.

DISCUSSION

The observations of Ingle *et al.* (1937, 1938), Sayers and Sayers (1945, 1947), Gray and Munson (1951), Abelson and Baron (1952), and Sydnor *et al.* (1955) demonstrate the inhibition of ACTH release following the administration of adrenocortical extracts, cortisone, and hydrocortisone. Porter and Jones (1956) observed that the injection of 6 mg. of hydrocortisone acetate per 100 gm. body weight prevented adrenal ascorbic acid depletion in the intact rat rollowing such acute stress as ether anesthesia and unilateral adrenalectomy. However, the injection of 4 ml. of whole plasma from the portal vessels resulted in adrenal ascorbic acid

[4] Mobilities are expressed as $cm.^2\ sec.^{-1}\ volt^{-1} \times 10^{-5}$ with standard deviations.

reduction of -30 ± 11.2 mg. per 100 gm. adrenal weight in similarly treated rats. This reduction did not occur in the hypophysectomized rat receiving an equal volume of portal vessel plasma or in the hydrocortisone-inhibited, intact rat receiving 4 ml. of carotid artery plasma. These observations as well as those presented here suggest that the site of inhibition by hydrocortisone is not the anterior lobe but some other center, possibly the hypothalamus, and that portal vessel plasma contains a substance which promotes the release of ACTH by the anterior pituitary.

Lyophilization of the portal plasma permits the injection of quantities equivalent to greater volumes of whole plasma. When lyophilized portal vessel plasma equivalent to 10 ml. of plasma was injected intravenously into hydrocortisone-inhibited, intact rats, the change in adrenal ascorbic acid concentration was -107 ± 12.9 mg. per 100 gm. adrenal weight; this response was significantly greater than that observed in similarly treated hypophysectomized rats or hydrocortisone-inhibited, intact rats receiving an equivalent volume of lyophilized carotid artery plasma ($P < 0.01$).

The observation that the active substance did not diffuse through a dialysis membrane during prolonged dialysis indicates that it is a large molecule, *e.g.* protein, or a smaller molecule bound to protein. In purification by alcohol fractionation (Cohn *et al.*, 1946) all of this substance present in portal vessel plasma as measured by its activity is contained in precipitate II+III with no activity residing in the remaining five fractions. Further purification is in progress.

The injection of commercial Pitressin into hydrocortisone-inhibited, intact rats caused reductions in adrenal ascorbic acid, confirming the findings of McCann and Brobeck (1954) and Guillemin and Hearn (1955). Briggs and Munson (1955), however, reported no change in adrenal ascorbic acid in the morphine-treated rat receiving a much smaller dose of Pitressin than those used in this investigation. It is of especial interest that Guillemin and Hearn (1955) found that purified arginine-vasopressin had no significant effect on the release of ACTH by the *in vitro* anterior lobe. Although blood pressure was not measured in this investigation, it was noted that injection of Pitressin caused marked blanching of the feet indicative of peripheral vasoconstriction; this was not present following injections of precipitate II+III equivalent to 10 ml. and 22 ml. of portal vessel plasma even though the latter gave a much greater ascorbic acid response. These observations would indicate that the substance in portal vessel plasma as well as that in Pitressin which promotes the release of ACTH by the anterior lobe is probably not identical with vasopressin.

SUMMARY

Hydrocortisone-inhibited, intact rats injected with an amount of lyophilized hypothalamico-hypophyseal portal vessel plasma equivalent to 10 ml. of whole plasma showed a decrease of -107 ± 12.9 mg. of ascorbic

acid per 100 gm. adrenal tissue. Similarly treated intact rats receiving an equivalent amount of lyophilized carotid artery plasma or hypophysectomized rats receiving an equivalent amount of lyophilized portal vessel plasma showed decreases of only 0 ± 2.8 and -14 ± 5.4 respectively.

The substance in portal plasma responsible for this ascorbic acid reduction is non-dialyzable and following low temperature alcohol fractionation of the plasma proteins is found to be present in a single fraction.

Similar activity was found to be present in commercial Pitressin as previously observed. The results indicate the active substance is either a large protein molecule or is bound to a large protein and is probably not identical with vasopressin.

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REFERENCES

ABELSON, D. AND D. N. BARON: *Lancet* **2**: 663. 1952.
BRIGGS, F. N. AND P. L. MUNSON: *Endocrinology* **57**: 205. 1955.
COHN, E. J., L. E. STRONG, W. L. HUGHES, JR., D. J. MULFORD, J. N. ASHWORTH, M. MELIN AND H. L. TAYLOR: *J. Am. Chem. Soc.* **68**: 459. 1946.
DEGROOT, J. AND G. W. HARRIS: *J. Physiol.* **111**: 335. 1950.
GANONG, W. F. AND D. M. HUME: *Endocrinology* **55**: 474. 1954.
GRAY, W. D. AND P. L. MUNSON: *Endocrinology* **48**: 471. 1951.
GREEN, J. D. AND G. W. HARRIS: *J. Endocrinol.* **5**: 136. 1947.
GREEN, J. D. AND G. W. HARRIS: *J. Physiol.* **108**: 359. 1949.
GUILLEMIN, R.: *Fed. Proc.* **14**: 65. 1955.
GUILLEMIN, R. AND W. R. HEARN: *Proc. Soc. Exper. Biol. and Med.* **89**: 365. 1955.
HARRIS, G. W. AND D. JACOBSOHN: *Proc. Royal Soc.* B. **139**: 263. 1952.
HINSEY, J. C. AND J. E. MARKEE: *Proc. Soc. Exper. Biol. and Med.* **31**: 270. 1933.
HUME, D. M. AND G. J. WITTENSTEIN: *Proc. 1st Clin. ACTH Conf. Ed.* by J. R. Mote, Philadelphia, Blakiston, 1950.
INGLE, D. J. AND E. C. KENDALL: *Science* **86**: 245. 1937.
INGLE, D. J., G. M. HIGGINS AND E. C. KENDALL: *Anat. Rec.* **71**: 363. 1938.
JACOBSOHN, D.: *Acta Endocrinol.* **17**: 187. 1954.
LAQUEUR, G. L., S. M. MCCANN, L. H. SCHREINER, E. ROSEMBERG, D. MCK. RIOCH AND E. ANDERSON: *Endocrinology* **57**: 44. 1955.
MCCANN, S. M.: *Am. J. Physiol.* **175**: 13. 1953.
MCCANN, S. M. AND J. R. BROBECK: *Proc. Soc. Exper. Biol. and Med.* **87**: 318. 1954.
MCCANN, S. M. AND K. L. SYDNOR: *Proc. Soc. Exper. Biol. and Med.* **87**: 369. 1954.
PORTER, J. C. AND J. C. JONES: *Endocrinology.* **58**: 62. 1956.
PORTER, R. W.: *Am. J. Physiol.* **172**: 515. 1953.
SAYERS, G. AND M. A. SAYERS: *Proc. Soc. Exper. Biol. and Med.* **60**: 162. 1945.
SAYERS, G. AND M. A. SAYERS: *Endocrinology* **40**: 265. 1947.
SAYERS, M. A., G. SAYERS AND L. A. WOODBURY: *Endocrinology* **42**: 379. 1948.
SYDNOR, K. L., R. BURKS AND D. BROWN: *Endocrinology* **56**: 204. 1955.
WISLOCKI, G. B. AND L. S. KING: *Am. J. Anat.* **58**: 421. 1936.
XUEREB, G. P., M. M. L. PRICHARD AND P. M. DANIEL: *Quart. J. Exper. Physiol.* **39**: 219. 1954.

THE DIURNAL VARIATION OF BLOOD LEUCOCYTES IN NORMAL AND ADRENALECTOMIZED MICE[1,2]

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FOR many years conflicting opinions have appeared in the literature concerning cyclic variation of the circulating leucocytes. Recently, a diurnal variation of large magnitude has been well established for the circulating eosinophils in a variety of animals, Halberg and Visscher (1950), Halberg (1953), Halberg, Visscher and Bittner (1953), Visscher and Halberg (1955), Louch, Meyer and Emlen (1953), Panzenhagen and Speirs (1953), and has been reported for the lymphocytes in the human by Elmadjian and Pincus (1946).

With the advent of intensive research on the pituitary and adrenal hormones, a marked effect of these hormones on the numbers of circulating leucocytes became evident. Dougherty and White (1943, 1944, 1947) and White and Dougherty (1945) observed an involution of lymphatic tissue and concomitant blood lymphopenia and neutrophilia following administration of these hormones. Pincus (1943) observed a diurnal variation in the urinary excretion of 17-ketosteroids in humans and Laidlaw *et al.* (1954) found a diurnal variation in urinary 17-hydroxycorticoids. Elmadjian and Pincus (1946), using the lymphopenic response as an indicator of adrenal cortical activity, found a diurnal variation of the blood lymphocytes which correlated with 17-ketosteroid excretion. Halberg, *et al.* (1953) and Visscher and Halberg (1955) reported that adrenalectomy appeared to abolish the diurnal cycle of the circulating eosinophils in mice.

Changes in the number of circulating lymphocytes and eosinophils have long been used as indicators of adrenal cortical activity and adrenalectomy appears to abolish the diurnal variation of the eosinophils. Therefore, a close correlation appears to exist between the numerical variations of the eosinophil, during the diurnal cycle, and adrenal cortical activity. A study of all the leucocytes of a single species during the diurnal cycle has not been reported. This work was undertaken to establish a correlative blood picture, during the diurnal cycle, for the various blood leucocytes of the mouse, and to determine the effect of adrenalectomy on these variations.

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MATERIALS AND METHODS

The animals used were inbred, male mice 12 to 16 weeks of age from a subline of the C-57, Black strain. The animals were kept in a thermostatically controlled room and fed *ad libitum* Purina dog checkers and wheat, supplemented with dried yeast and cod liver oil. The animals were left undisturbed for as long as possible previous to taking blood samples and no mouse was used which was known to have been disturbed by any kind of stress for at least 24 hours before the blood samples were taken. Each animal was used for only one determination.

Tail vein blood was utilized for all counts and total white cell and eosinophil counts were done in the same chamber using a modification of the diluting fluid described by Randolph and Stanton (1945). Eosinophil values were also determined from the differential counts. Differentials were obtained by counting 300 cells on blood smears stained with Wrights-Giemsa. Blood basophils were not seen in the entire series of blood samples and monocytes were seen only occasionally and are not included in the following data. Adrenalectomy was carried out under light ether anesthesia by the dorsal approach and the animals were allowed to recover for 2 days following the operation. In the normal group, blood samples were taken at 3 hour intervals over a 24 hour period. In the adrenalectomized group, blood samples were obtained at 2 periods of the day which corresponded to the times when the leucocyte levels of normal intact animals were at their highest and lowest values. Counts were taken at intervals of 2, 5, 9 and 14 days after adrenalectomy. Hematocrit values were obtained from normal animals at the same two times of day using a micro-hematocrit technique.

EXPERIMENTAL OBSERVATIONS

A—*Normal Diurnal Cycle*

1. *Absolute leucocyte counts*

The results (Fig. 1 and Table 1) show a marked, cyclic diurnal variation in the numbers of all the leucocytes of the mouse. The cycle is characterized

TABLE 1. DIURNAL VARIATION OF THE BLOOD LEUCOCYTES IN TAIL VEIN BLOOD OF C-57 BLACK MICE 12 WEEKS OF AGE

Time of day	Number of animals	Total WBC	Eosinophils				Absolute neutrophils		Absolute lymphocytes	
			Chamber		Smear					
		/CMM	Total*	%†	Total	%	Total	%	Total	%
06:00 A.M.	20	14,016 ±1010	412 ± 54	2.87 ±.28	313 ± 51	2.28 ±.30	1406 ±155	10.87 ±1.39	12,298 ± 964	86.86 ±1.56
09:00 A.M.	20	19,029 ± 268	978 ±102	5.07 ±.46	903 ±116	4.74 ±.55	2381 ±198	12.71 ±1.07	15,744 ± 771	82.55 ±1.16
12:00 A.M.	20	20,692 ±1397	686 ± 55	3.39 ±.26	651 ± 82	3.13 ±.38	2180 ±249	10.45 ± .90	17,870 ±1212	86.45 ±1.11
15:00 3 P.M.	20	21,149 ± 700	978 ± 83	4.65 ±.40	877 ±102	4.15 ±.48	1574 ±185	7.39 ± .73	18,701 ± 645	88.47 ± .99
18:00 6 P.M.	20	16,110 ± 840	449 ± 42	2.79 ±.22	357 ± 43	2.20 ±.23	1568 ±205	9.96 ±1.16	14,185 ± 798	87.84 ±1.14
21:00 9 P.M.	20	7,082 ± 528	214 ± 30	3.00 ±.33	258 ± 43	3.71 ±.52	755 ± 55	11.30 ± .88	6,070 ± 501	85.00 ±1.21
24:00 12 P.M.	20	10,938 ±1200	201 ± 26	1.90 ±.18	126 ± 19	1.26 ±.20	1201 ±187	11.96 ±1.43	9,615 ±1151	86.80 ±1.43
03:00 A.M.	20	15,000 ± 750	492 ± 51	3.23 ±.26	347 ± 43	2.34 ±.27	1580 ±198	10.46 ±1.11	13,073 ± 683	87.20 ±1.19

* Total counts/cmm of tail vein blood ± standard error.
† % of total WBC ± standard error.

by an increase of all the leucocytes throughout the morning hours to a peak reached in the early afternoon. A decrease then occurs reaching a low in the late evening with a subsequent return to morning values.

The curves for the total white cell counts and total lymphocyte counts are almost identical, since the lymphocytes constitute a large majority of

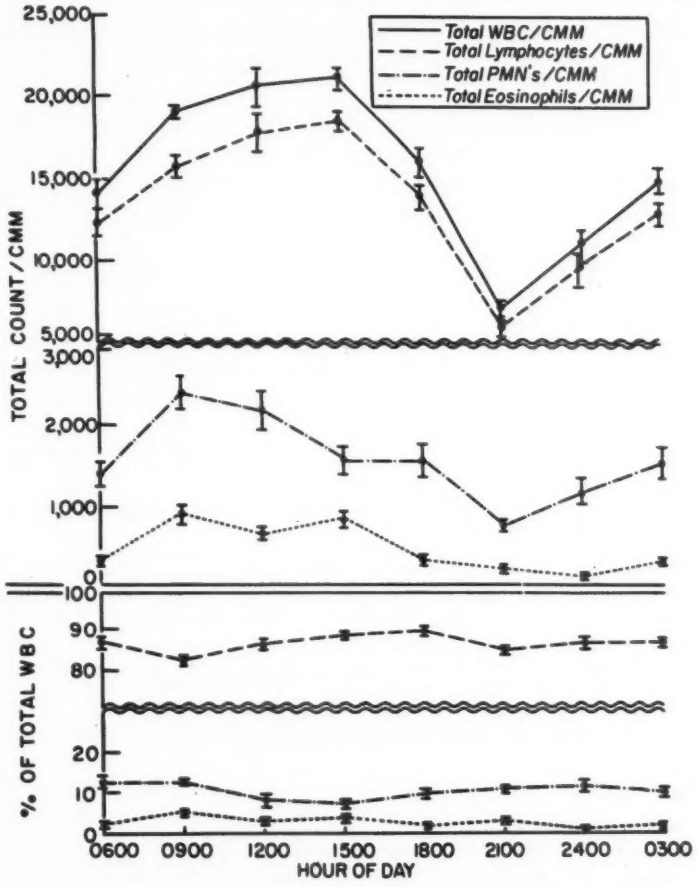

FIG. 1. Diurnal variation of blood leucocytes from tail vein blood of C-57 Black mice.

the leucocytes present in mouse blood. These curves present a peak which is reached between 12:00 A.M. and 3:00 P.M., and a low reached at approximately 9:00 P.M. The decrease is a rapid one, taking place during a period of 6 hours, with a more gradual return to the afternoon high.

The neutrophil or PMN curve is very similar except the high peak is reached sooner, between 9:00 A.M. and 12:00 A.M. The low occurs at the same period as that for the total WBC and total lymphocytes. The increase

and decrease in neutrophils is more symmetrical; each takes place over a period of approximately 12 hours duration.

The eosinophil curve is also quite similar with the exception that the high peak, being reached at approximately the same time as that of the neutrophil, is maintained until the time that the decrease occurs for the lymphocytes at 3:00 P.M. The low for the eosinophils occurs slightly later than that for the neutrophils and lymphocytes being reached at approximately 12:00 P.M. The increase and decrease are symmetrical; each taking approximately 12 hours.

The eosinophil values were obtained by both the direct chamber count and by a 300 cell differential count on the stained blood smear, in order to make a comparison of the relative accuracy of the two methods. The results (Fig. 2) gave excellent correlation between the two methods. The chamber

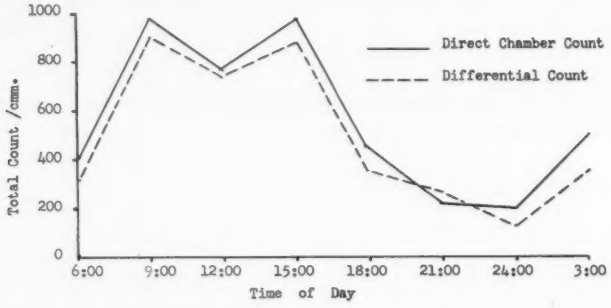

FIG. 2. The diurnal variation of the circulating eosinophils in tail vein blood of C-57 Black mice as determined by both the direct chamber count and 300 cell differential count.

count appeared to be slightly more accurate since the standard errors were lower in most cases.

2. *Leucocyte values as per cent of the total WBC*

When the number of eosinophils, neutrophils, and lymphocytes are expressed as per cent of the total WBC and compared for each of the time intervals tested, only minor changes in percentages occur over the 24 hour cycle for each cell type (Fig. 1, Table 1). The high and low values of each curve when analyzed statistically do show a significant difference between the two points (P .01). The range between the high and low percentages for each cell type is 5 or 6%. This indicates that despite a large change in the numbers of leucocytes over the 24 hour cycle, the percentages of each cellular element remain fairly constant.

3. *Hematocrit*

Because of the high variation in the leucocyte numbers observed during the diurnal variation, hematocrit values were obtained during the high

TABLE 2. HEMATOCRIT

High period		Low period	
No. animals	Hematocrit	No. animals	Hematocrit
10	55.98% ±0.99%	10	50.71% ±1.23%

and low points of the total leucocyte curve to determine if the changes might be due entirely or partly to hemo-dilution. The results shown in Table 2 indicate that a small difference does exist between the two periods. The difference between the two values was 5% which was found to be significant (P .01). This amount of hemodilution would not be enough to account for the large variation in leucocyte numbers and, therefore, plays only a minor part in the diurnal variation.

B—*The Effect of Adrenalectomy on the Diurnal Variation of Blood Leucocytes in Mice*

Since the numerical distributions of the circulating leucocytes have been shown to be markedly altered by the secretions of the adrenal gland, an experiment was performed to determine if the variations of the other leucocytes, which are observed during the diurnal cycle, are affected by adrenalectomy in the same manner as that reported for the eosinophils.

1. *Absolute leucocyte counts after adrenalectomy*

It can be seen from Fig. 3, that a very significant difference exists between high and low counts in the intact mouse (P .001). Two days after adrenalectomy the only semblance of a day-night difference which remains is in the case of the neutrophils, which appear from the literature to be the cell least regulated by adrenal hormones, and this difference is present only at the 2 day post-operation period (P .05). The high and low difference is no longer present for the other types of leucocytes two days after adrenal-

TABLE 3. THE EFFECT OF ADRENALECTOMY ON THE LEVEL OF BLOOD LEUCOCYTES TAKEN DURING THE *high* PERIOD OF THE NORMAL DIURNAL CYCLE IN MICE

Time after adrenalectomy	Number of animals	Total WBC	Total eosinophils				Total neutrophils		Total lymphocytes	
			Chamber		Smear					
		Absolute	Absolute	%	Absolute	%	Absolute	%	Absolute	%
Normal intact	20	21,149 ± 700	978 ± 83	4.65 ± .40	877 ±102	4.15 ± .48	1574 ±185	7.39 ± .73	18,701 ± 645	88.47 ± .97
2 days post .op.	15	21,472 ±1010	554 ± 48	2.56 ± .15	460 ± 67	2.12 ± .25	3402 ±369	16.06 ±1.73	17,604 ± 941	81.79 ±1.74
5 days post op.	13	26,106 ±1411	1283 ±219	4.86 ± .65	717 ±110	2.76 ± .44	2698 ±297	10.80 ±1.30	22,698 ±1408	86.48 ±1.30
9 days post. op.	11	22,700 ±1584	765 ± 33	3.56 ± .33	556 ±101	2.69 ± .57	2359 ±458	10.14 ±1.49	19,783 ±1387	87.16 ±1.74
14 days post op.	8	22,431 ±1716	702 ± 68	3.15 ± .29	362 ± 68	1.62 ± .31	1538 ±215	7.23 ±1.23	20,530 ±1728	91.15 ±1.44

TABLE 4. THE EFFECT OF ADRENALECTOMY ON THE LEVEL OF BLOOD LEUCOCYTES TAKEN DURING THE *low* PERIOD OF THE NORMAL DIURNAL CYCLE IN MICE

Time after adrenalectomy	Number of animals	Total WBC	Total eosinophils				Total neutrophils		Total lymphocytes	
			Chamber		Smear					
		Absolute	Absolute	%	Absolute	%	Absolute	%	Absolute	%
Normal Intact	20	10,938 ±1200	201 ± 26	1.90 ± .18	126 ± 19	1.26 ± .20	1201 ±187	11.96 ±1.43	9615 ±1151	86.80 ±1.43
2 days post op.	15	18,997 ±1525	520 ± 95	2.61 ± .36	329 ± 77	1.73 ± .35	2351 ±308	13.72 ±1.97	16,315 ±1598	84.54 ±2.03
5 days post op.	12	23,419 ±2112	728 ± 73	3.24 ± .32	664 ±107	3.12 ± .53	2962 ±584	13.57 ±2.60	19,721 ±2127	83.07 ±2.88
9 days post op.	14	20,547 ±1720	857 ±117	4.23 ± .50	565 ± 94	2.94 ± .57	1692 ±166	8.83 ±1.03	18,289 ±1708	88.23 ±1.30
14 days post op.	8	23,041 ±1805	878 ±158	3.87 ± .77	553 ±114	2.34 ± .50	1901 ±260	8.44 ±1.18	20,585 ±1692	89.22 ±1.25

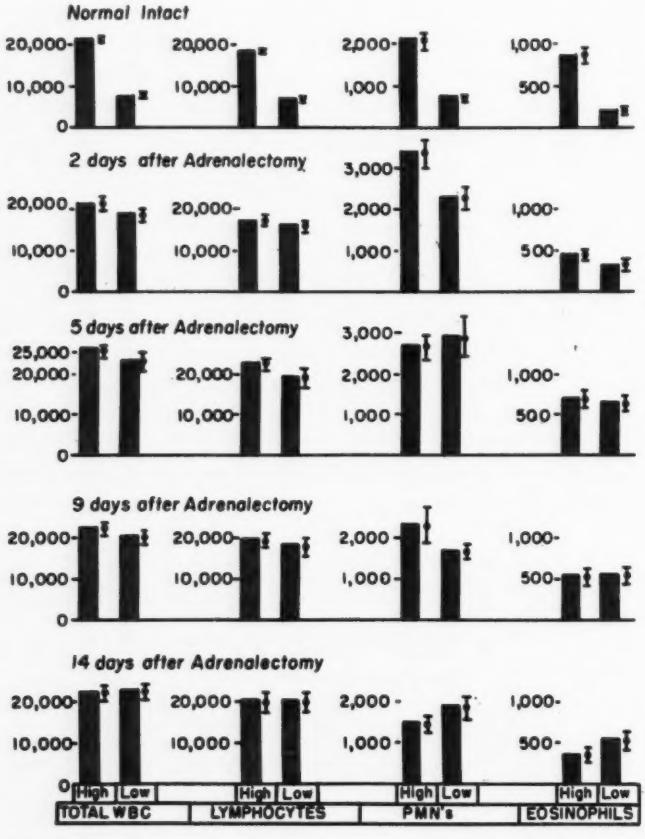

FIG. 3. The effect of adrenalectomy on the diurnal variation of blood leucocytes from tail vein blood of mice.

ectomy (Fig. 3, Tables 3 and 4). A significant neutrophilia is present at both the high and low periods when compared to normal-values (P .01). A significant eosinopenia is found at the high period (P .01) but not at the low period. A significant lymphocytosis is found at the low period (P .01) but not at the high period of the cycle when compared to normal values. The total WBC count shows a significant leucocytosis at the low period (P .01) but the high period has values within the normal limits. All the changes in leucocyte numbers which occur at the high and low periods 2 days after adrenalectomy, when compared with normal values, are changes which bring about the abolishment of the high-low differences of the normal diurnal cycle.

The leucocyte counts taken five days after adrenalectomy show leucocytosis at both the high and low periods which is significantly above that found during the high and low periods in normal mice (P .01). The leucocytosis is produced by an increase in number of lymphocytes at both high and low periods and a neutrophilia at both periods which has been carried over from the second post-operative day (P .01 in both cases). The eosinophils show a slight increase at both periods to a level which is intermediate in position between the high and low levels of normal mice. Any semblance of a diurnal cycle is now absent since the high and low values for each of the three types of leucocytes show no significant difference.

The leucocyte counts taken nine days after adrenalectomy show, at both high and low periods, a return to the values found during the high period of the diurnal cycle in intact animals. An exception is made for the eosinophils which at both periods remain at a level intermediate between normal diurnal high and low values. There is no indication of a return of the diurnal cycle for any of the cell types, since there is no significant difference between the values obtained at the high and low periods for each type.

The leucocyte counts taken 14 days after adrenalectomy show essentially the same pattern as those at nine days. The counts are all within the limits of the values obtained at the high period of the normal diurnal cycle except those of the eosinophils which remain at an intermediate level.

The most striking change in leucocyte numbers of adrenalectomized mice is the elimination of diurnal cyclic variations between high and low periods of the normal cycle. This is produced by a rapid increase in the number of leucocytes found at the low period to levels within the limits found during the high period.

A lymphocytosis after adrenalectomy is not a constant finding at each period tested when compared with the high period of the normal cycle. However, if all the counts obtained over the 14 day post-operative period are pooled and compared with the normal intact values obtained at the high period of the normal cycle, a slight lymphocytosis if found (P .05).

2. *Leucocyte counts expressed as per cent of the total WBC after adrenalectomy*

The leucocyte counts, when expressed as per cent of the total WBC, show only minor changes over the entire 14 day post-adrenalectomy period (Tables 3 and 4).

The lymphocytes and neutrophils have a range of 8% between high and low values at any period. The eosinophils have a range of 4% between the two extreme values. The largest difference occurs at the two-day period, when a neutrophilia is present in both absolute numbers and in per cent of total WBC. The percentage increase in neutrophils occurs at the expense of the lymphocytes which show a slight percentage lymphopenia at this time. The slight lymphocytosis in absolute numbers which occurs over the 14 day period is also reflected in a small percentage increase of the lymphocytes; however, this increase is not very striking.

DISCUSSION

The results of these experiments confirm the work of others in that a marked diurnal variation is observed in the circulating eosinophils of mice, but the results also show that the eosinophils are not the only cells which undergo these marked diurnal fluctuations. The lymphocytes and neutrophils show changes in number which are very similar in time and magnitude (Fig. 1). The mouse is a nocturnal animal with its active period at night and inactive period during the day. The numbers of leucocytes which are found in the tail vein blood are 3 to 4 times as great during the inactive period around 3:00 P.M. as they are during the active period around 9:00 P.M. It is apparent that in any experiments which measure changes in the number of blood leucocytes a close correlation must be maintained between the times that blood counts are obtained in both control and experimental animals; or the difference observed between control and experimental groups will reflect not only the effect of the experimental procedure but also the normal physiological variations which are observed during the diurnal cycle. Since a change in number of leucocytes in the ratios of 3 and 4 to 1 is larger than the experimental differences reported in many papers in the literature, the influence of the time of day becomes a factor of major importance.

The correlation between the high leucocyte counts obtained during the resting period and low leucocyte levels obtained during the active period, with adrenal functions appears excellent. Ingle, *et al.* (1952) have shown that physical activity is accompanied by an increased requirement of adrenal hormones and it has been shown repeatedly that increased amounts of adrenal hormones are accompanied by a decrease in lymphocytes and eosinophils in the circulating blood. These conditions are observed during the active period of the normal diurnal cycle. A complicating factor appears, however, to this convenient explanation. In many of the reports in

the literature in which an increased amount of adrenal secretion has been elicited endogenously or supplied exogenously, an increase in the number of neutrophils has been observed. This does not appear consistent with the decrease in neutrophils observed in these experiments during the active period of the diurnal cycle (Fig. 1). The degree of neutrophilia reported has been variable and not consistently measurable, however. The conditions under which the increased adrenal secretion has been obtained are almost invariably ones in which stress is the prominent factor; or when hormone has been administered, it is almost always in amounts which would not be found except under conditions of stress. In the experiments reported in this paper, scrupulous care was taken to avoid conditions of stress. It is possible that the neutrophil reaction may be different under stress than in more normal physiological conditions. It is appropriate to mention here that the stress lymphocytes, described by Dougherty and Frank (1953) and Frank and Dougherty (1953), and the neutrophils had curves during the normal diurnal cycle which were almost identical in spite of a decrease in absolute numbers during the first part of the active period.

Despite large variations in the absolute numbers during the normal diurnal cycle, the percentages of the various leucocytes present in tail vein blood showed only small variations. The percentage values in this case appear to be of definite value in determining normal physiological conditions as opposed to those of stress since many papers have reported percentage variations as well as changes in absolute numbers of leucocytes under conditions of stress. In normal mice it appears that the usual physiological amounts of hormone do not appreciably alter the percentage values of the circulating leucocytes; percentage variations appear to occur only when the adrenal hormone secretions exceed that found under normal physiological conditions. The absolute numbers of leucocytes under carefully controlled conditions appear to offer an accurate measurement of the amounts of hormone involved because of the magnitude of the variations which occur.

Halberg (1953) has reported that adrenalectomy appeared to abolish the diurnal variation of the circulating eosinophils. The experiments presented here confirm this observation. The results also show that the diurnal variation of all leucocyte types is abolished after adrenalectomy. Two days after the operation only the neutrophils show a significant difference between day and night values and this difference is very small. Five days after adrenalectomy there is no significant difference between day and night values of any leucocyte type and the diurnal variation is not reconstituted over a 14 day period.

The characteristic trend of changes in the numbers of leucocytes present in mouse blood after adrenalectomy is an increase to the levels present at the high period of the normal diurnal cycle. The eosinophils stop at levels

intermediate between the normal high and low values while the lymphocytes increase to values only slightly higher than the levels found at the normal high period. The degree of lymphocytosis occurring after adrenalectomy, which has been reported in the literature, appears to depend on the time of day that control counts are made on intact animals. If control counts are made during a period of the diurnal cycle in which the lymphocytes are at a low level, a large difference between control and post-adrenalectomy values will be obtained. If control counts are obtained at a high period of the cycle, the difference will be very small. The same results may be obtained for the other leucocyte types.

SUMMARY

In the experiments just presented, the normal variations in the absolute number and percentages of the blood leucocytes over a 24 hour period were studied in intact and adrenalectomized mice.

A diurnal variation of large magnitude occurs in the number of blood leucocytes obtained from the tail vein blood of mice. The diurnal cycle is characterized by high leucocyte levels during the late morning and early afternoon which is the least active period in this nocturnal animal, and by low levels during the late evening which is the most active period.

Although large variations were observed for the absolute numbers of leucocytes, only minor variations were observed in the percentages of leucocytes present in tail vein blood during the normal diurnal cycle.

Hemodilution or hemoconcentration does not appear to be a major factor in the leucocyte variations. Although a small day and night variation in hematocrit was observed, it was not large enough to account for the magnitude of the leucocyte variations.

Adrenalectomy appears to abolish the diurnal variation of the circulating leucocytes since no day and night differences were observed over a 14 day period after the adrenal glands were removed.

The leucocyte counts after adrenalectomy assume levels which are characteristic for the high period of the normal diurnal cycle. A slight exception is noted for the eosinophils which maintain a level intermediate between normal high and low values and for the normal lymphocytes which show a slight lymphocytosis at these two periods.

The leucocyte variations during the diurnal cycle in mice correlate well with other indices of adrenal function. Since adrenalectomy abolishes these variations, it appears that they are mediated through the action of the adrenal hormones. Diurnal variations for eosinophils and total lymphocytes have been observed in humans, and the eosinophil variations have been reported to be markedly reduced in Addison's disease and panhypopituitarism. More experiments are needed, however, to work out the details of human diurnal variations.

These results illustrate that in experimental work which is concerned

with measuring changes in the number of circulating leucocytes, the interpretations of results must take into consideration normal diurnal variations or the data will reflect not only experimental differences but normal diurnal changes as well.

REFERENCES

DOUGHERTY, T. F. AND A. WHITE: *Proc. Soc. Exper. Biol. & Med.* **53**: 132. 1943.
DOUGHERTY, T. F. AND A. WHITE: *Endocrinology* **35**: 1. 1944.
DOUGHERTY, T. F. AND A. WHITE: *J. Lab. & Clin. Med.* **32**: 584. 1947.
DOUGHERTY, T. F. AND J. A. FRANK: *J. Lab. & Clin. Med.* **42**: 530. 1953.
ELMADJIAN, F. AND G. PINCUS: *J. Clin. Endocrinol.* **6**: 287. 1946.
FRANK, J. A. AND T. F. DOUGHERTY: *J. Lab. & Clin. Med.* **42**: 538. 1953.
HALBERG, F.: *J. Lancet.* **73**: 20. 1953.
HALBERG, F. AND M. B. VISSCHER: *Proc. Soc. Exper. Biol. & Med.* **75**: 846. 1950.
HALBERG, F., M. B. VISSCHER AND J. J. BITTNER: *Am. J. Physiol.* **174**: 109. 1953.
INGLE, D. S., J. E. NEZAMIS AND E. H. MORLEY: *Endocrinology* **50**: 1. 1952.
LAIDLAW, C., D. JENKINS, W. J. REDDY AND T. JAKOBSON: *J. Clin. Invest.* **33**: 950. 1954.
LOUCH, C., R. F. MEYER AND J. T. EMLEN: *Proc. Soc. Exper. Biol. & Med.* **82**: 668. 1953.
PANZENHAGEN, H. AND R. SPIERS: *Blood.* **8**: 536. 1953.
PINCUS, G.: *J. Clin. Endocrinol.* **3**: 195. 1943.
RANDOLPH, T. F. AND C. L. STANTON: *Am. J. Clin. Path.* **15**: 17. 1945.
VISSCHER, M. B. AND F. HALBERG: *Ann. N. Y. Acad. Sci.* **59**: 834. 1955.
WHITE, A. AND T. F. DOUGHERTY: *Endocrinology* **36**: 16. 1945.

THE INTERCONVERSION OF ESTRONE AND ESTRADIOL IN THE PERFUSED HUMAN PLACENTA[1,2]

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THE human placenta is a relatively abundant source of estrogens. The determination of the concentration of estrone, estradiol and estriol in that organ has been the subject of several investigations (Huffman, Thayer and Doisy, 1940; Mitchell, 1952; Diczfalusy, 1953). More recently, the metabolism of estrogens in the placenta has also been studied. Pearlman, Cerceo and Thomas (1954) using homogenates and Ryan and Engel (1953) using slices demonstrated the capacity of the human placenta to convert estradiol to estrone. The latter authors further found that under their experimental conditions there was no conversion of estrone to estradiol by term placenta and only minimal conversion by early placenta.

The present investigation was undertaken to examine the question of interconversion of estrone and estradiol in the perfused human placenta. The use of radioactive estrogens made it possible to work at very low concentrations.

METHODS

Perfusion Procedure: A human placenta was immersed in cold Tyrode's solution immediately after a normal delivery. A cotyledon was prepared for perfusion by cannulating an artery and cutting the tributary vein (Chesley and McFaul, 1949). Usually 25 minutes elapsed between the delivery of the placenta and perfusion. The perfusion medium was 160 ml. of human plasma containing 25 mg. of terramycin, 1.5 mg. of streptomycin and 1 mg. of penicillin. The plasma was obtained from outdated AB blood. It contained the standard citric acid and dextrose solutions. It was cycled at a rate of 30–40 ml./min. while being continuously oxygenated. About 5 minutes after attaching the placenta to the apparatus, estrone-16-C^{14} [4] or 17β-estradiol-16-C^{14} (Levitz, 1953) dis-

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[2] Presented in part at the 39th Annual Meeting of the Federation of the American Societies for Experimental Biology, April 12, 1955 in San Francisco.

[3] Lowell M. Palmer Senior Fellow.

[4] Estrone-16-C^{14} was purchased from Charles C. Frosst and Company, Montreal, Canada.

solved in propylene glycol was added to the perfusate. Perfusions were carried out at 37° C for 35 minutes (7 cycles) and for 4 hours (48 cycles). In the 4 hour experiments, samples were also taken at 35 minutes. Both perfusate and placenta were analyzed for radioactive estrone, estradiol and estriol by the isotope dilution technique.

In the experiments using perfused plasma, the plasma was first perfused through the placenta for 2 cycles. Approximately $\frac{1}{4}$ of the perfusate (40 ml.) was transferred to an Erlenmeyer flask and incubated with either radioactive estrone or estradiol for 35 minutes at 37° C. Analysis for radioactive estrone and estradiol followed. In control experiments, plasma that had not been previously perfused was similarly treated. To the plasma, 4 ml. of whole blood per 100 ml. of plasma was added to approximate the small concentration of red cells washed from the placenta into the perfusate.

Analytical Procedure: (a) Estrone. A 15 ml. aliquot of the perfusate was diluted with 35 ml. of normal saline solution and extracted continuously with ether for 60 hours. One hundred milligrams of estrone carrier[5] was added to the ether flask prior to the extraction. The ether was washed with 9% sodium bicarbonate and extracted with 2N sodium hydroxide. The alkali extract was acidified and reextracted with ether. The estrone so isolated was submitted to the Girard reaction (Girard and Sandulesco, 1936). The ketonic fraction was dissolved in benzene and placed on a column, 14 mm. in diameter, containing 9 gm. of silica gel. The column was eluted with a 2% solution of methanol in benzene. Estrone appeared in the fourth or fifth 20 ml. fraction. It was crystallized from 95% ethanol to constant specific activity. To demonstrate the purity of the product some samples were converted to the benzoate (Butenandt and Goergens, 1937). In no case did a loss of radioactivity accompany this transformation.

In some instances, continuous extraction of the perfusate with ether was replaced by precipitating the plasma proteins in 300 ml. of 1:1 acetone-ethanol containing 100 mg. of estrone carrier. The filtrate was taken nearly to dryness and extracted with ether. The ether extract was further processed as described above. Similar results were obtained with both methods.

(b) Estradiol and Estriol. One milligram of estriol[6,7] and 100 mg. of estradiol were used as carriers. The analytical procedure was the same as that described for estrone up to column chromatography except that estradiol and estriol appeared in the non-ketonic fraction in the Girard reaction. The column was eluted with 200 ml. of 2% methanol in benzene. Estradiol appeared in the fifth and sixth 20 ml. fractions. When the concentration of the methanol was increased to 4%, estriol was eluted in the sixth to tenth 20 ml. fractions. An additional 20 mg. of estriol was added to the fractions containing the estriol as revealed by a color test (Bachman, 1939). Crystallization from acetonitrile to constant specific activity followed. Estradiol was crystallized from ethanol to constant specific activity and converted to the dibenzoate.

(c) Placental Estrogens. Immediately after the conclusion of the perfusion, the placenta was extracted in the Waring Blender with three 250 ml. portions of 1:1 acetone-ethanol. Carrier was added to a 200 ml. aliquot of the extract. The solution was evaporated nearly to dryness and extracted with ether. The ether extract was treated as described for the perfusate.

Counting: After isolation and purification of the estrogens, accurately weighed samples of about 0.2 mg. dissolved in 0.5 ml. of ethanol were placed on aluminum planchets and dried under an infra-red lamp. The radioactivity was determined in the D-46A Nuclear Flow Gas Counter and the counts per minute (c.p.m.) were corrected to infinite thinness.

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[5] The authors are indebted to the Schering Company for generous gifts of estrone and estradiol.

[6] The authors are indebted to Parke Davis and Company for generous gifts of estriol.

[7] Because of the insolubility of estriol, only 1 mg. was added at this time.

The per cent conversion of the perfused material was calculated according to the following formula:

$$\frac{\text{c.p.m./mg. in final product} \times \text{mg. carrier}}{\text{Total c.p.m. in precursor}} \times \frac{\text{ml. perfusate}}{\text{ml. aliquot}} \times 100.$$

The estimated accuracy of the reported results is $\pm 10\%$.

RESULTS

Perfusion of the placenta with 17β-estradiol-16-C^{14} for 35 minutes resulted in a 58% conversion to estrone, $45 \pm 5\%$ of which was in the perfusate and $13 \pm 3\%$ in the placenta (Table 1). The recovered estradiol

TABLE 1. ESTRADIOL-ESTRONE INTERCONVERSION IN PERFUSED HUMAN PLACENTA

Precursor, μg.	Wet weight placenta, gm.	35 minutes				4 hours			
		Perfusate		Placenta		Perfusate		Placenta	
		Estradiol	Estrone	Estradiol	Estrone	Estradiol	Estrone	Estradiol	Estrone
A—Estradiol									
307	96	25	49			41	10		
422	107	14	50	19	19				
428	110	17	35	26	10				
458	135	13	48	27	12				
644	112	13	49			30	17	4	19
816	109	19	40	28	12				
1029	50	20	47						
B—Estrone									
534	170	17	33			32	3	26	20
667	95	10	46	16	11				
799	97	18	39	14	22				
950						31	22	10	32

Amount of recovered estrogen is expressed as per cent of starting material.

totaled 42%, $17 \pm 3\%$ in the perfusate and $25 \pm 3\%$ in the placenta. Thus the ratio of estrone to estradiol in the perfusate was 2.5 to 1 and in the placenta, it was 1 to 2. Since the placenta includes an unmeasured amount of perfusate contained in blood vessels, the estrone-estradiol ratio in placenta is actually lower than 1:2.

Similar estrone-estradiol ratios were obtained in the perfusate when estrone-16-C^{14} was the starting material. There is insufficient data for comparison of the placental ratios.

TABLE 2. ESTRADIOL-ESTRONE INTERCONVERSION BY PLACENTAL PERFUSATE

Precursor	Concentration, μg./ml.	Estradiol	Estrone
Perfusate Plasma*			
Estradiol	2.7	38	59
Estrone	2.3	15	81
Control Plasma†			
Estradiol	1.5	107	0
Estrone	2.6	1.5, 0	107

Amount of recovered estrogen is expressed as per cent of starting material.

* The plasma was cycled twice through the placenta, then radioactive estrogens were incubated in aliquots of the perfusate.

† To approximate the red blood cell concentration in the experiments with perfused plasma, 4 ml. of freshly drawn blood was added to 100 ml. of blood bank plasma.

The 4-hour data are less consistent but it is evident that the estrone-estradiol ratio in the perfusate is lower, while that in the placenta is higher than that found at 35 minutes. This was true regardless of which estrogen was perfused.

Plasma, prior to perfusion, was unable to interconvert estrone and estradiol. However, after 2 cycles through the placenta, the perfusate alone could perform this interconversion (Table 2).

No radioactive estriol could be detected in the placenta or perfusate in any of the experiments.

DISCUSSION

The data presented indicates that in the perfused human placenta estrone and estradiol equilibrate. After 35 minutes a reproducible ratio of estrone to estradiol is observed in the perfusate which is independent of which precursor is used and, within the limits studied, of the amount of precursor and the weight of the perfused tissue.

Ryan and Engel (1953) reported that term placental slices convert estradiol to estrone but do not effect the reverse reaction. It is not simple to explain this discrepancy in results, but it may be significant that these investigators used a substrate to tissue ratio 2000 times that used in the perfusion experiments.

The ratio of estrone to estradiol in placental tissue differs significantly from that in the perfusate. This may be a result of the preferential transfer of one of the estrogens across the cell membrane or may be dependent on different concentrations of the reduced and oxidized forms of cofactors, in the placenta and in the perfusate; e.g. DPN (Pearlman, Cerceo and Thomas, 1954). These factors may also explain the observation that the individual ratios change markedly with prolonged perfusion.

There is evidence (Stewart, 1951; Levitz, Condon and Dancis, 1955) that the human placenta synthesizes estrogens. It would appear from the results reported that the placenta can also control the level of estrogenic activity by controlling the ratio of estrone to the more physiologically active estradiol. In addition, estrone inhibits the activity of estradiol as measured by the stimulation of uterine growth in the castrated rat (Hisaw, Velardo and Goolsby, 1954).

It must be kept in mind that the translation of our perfusion experiments on the isolated placenta to the conditions *in vivo* must be done with reservations. It would seem reasonable to conclude that the placenta is able to maintain an equilibrium between estrone and estradiol, but the ratios determined in these experiments may not reflect those present *in vivo*. The observation that the perfusate gains the capacity to interconvert estrone and estradiol after only 2 cycles through the placenta must also be approached with the same caution. Whether this is a result of the artificial conditions in the perfusion experiments or actually represents a

mechanism of controlling the estrone-estradiol ratio in the circulating blood is under investigation.

SUMMARY

The interconversion of C^{14} labeled estrone and estradiol was studied in the perfused human placenta. The perfusate and the placenta were analyzed for radioactive estrogens after placentas had been perfused for 35 minutes and for 4 hours.

Reproducible ratios of estrone to estradiol were observed in the perfusate. They were independent of which precursor was used and, within the limits studied, of the amount of precursor and the weight of the perfused tissue.

The estrone to estradiol ratios in perfusate and placenta changed with prolonged perfusion. The ratios in the perfusate differed from those in the placenta at 35 minutes and at 4 hours.

After being perfused through the placenta for 2 cycles, the perfusate gained the capacity to interconvert estrone and estradiol.

There was no detectable conversion of estradiol or estrone to estriol.

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REFERENCES

BACHMAN, C.: *J. Biol. Chem.* **131**: 463. 1939.

BUTENANDT, A. AND C. GOERGENS: *J. Physiol. Chem.* **248**: 129. 1937.

CHESLEY, L. C. AND I. E. MCFAUL: *Am. J. Obst. and Gynec.* **58**: 159. 1949.

DICZFALUSY, E.: *Acta Endocr.*, Copenhagen, 12, Suppl. No. 12. 1953.

GIRARD, A. AND G. SANDULESCO: *Helv. Chim. Acta* **19**: 1095. 1936.

HISAW, F. L., J. T. VELARDO AND C. M. GOOLSBY: *J. Clin. Endocr. and Metab.* **14**: 1134. 1954.

HUFFMAN, M. N., S. A. THAYER AND E. A. DOISY: *J. Biol. Chem.* **133**: 567. 1940.

LEVITZ, M.: *J. Am. Chem. Soc.* **75**: 5352. 1953.

LEVITZ, M., G. P. CONDON AND J. DANCIS: *Fed. Proc.* **14**: 245. 1955.

MITCHELL, F. L.: *Nature* **170**: 621. 1952.

PEARLMAN, W. H., E. CERCEO AND M. THOMAS: *J. Biol. Chem.* **208**: 234. 1954.

PEARLMAN, W. H., E. CERCEO AND M. THOMAS: *Fed. Proc.* **13**: 272. 1954.

RYAN, K. J. AND L. L. ENGEL: *Endocrinology* **52**: 287. 1953.

STEWART, H. L. JR.: *Am. J. Obst. and Gynec.* **61**: 990. 1951.

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IODINE METABOLISM IN SURVIVING HUMAN AND CANINE THYROID SLICES[1]

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A METHOD of studying iodine metabolism in surviving thyroid slices of animals by adding radioiodine to a fixed volume of incubating medium was developed by Morton and Chaikoff (1942, 1943) and by Schachner, Franklin and Chaikoff (1944). With minor modifications, this method has been employed by all subsequent workers. Freinkel and Ingbar (1955a, 1955b), Morton, Chaikoff and Rosenfeld (1944) and Fawcett and Kirkwood (1953) have established that stable iodine added to the medium interferes with accumulation of radioiodine in the thyroid slice.

During a study of the behavior of canine and human thyroid slices by this method, it was found that variable but appreciable quantities of I^{127} of the thyroid slice entered the medium. The quantity of I^{127} lost from the tissue was sufficient to lower significantly the specific activity of the iodine in the medium, thus interfering with the accumulation of I^{131} by the slice.

MATERIAL AND METHODS

Histologically normal thyroid tissue was obtained by biopsy from seven clinically euthyroid patients during operations on the neck. Tissue from three patients with exophthalmic goiter who were prepared in the customary fashion for operation with strong iodine solution (Lugol's solution) was secured at thyroidectomy. Normal thyroid tissue was also obtained from 10 mongrel dogs maintained on Fromm dog food containing 0.5% iodized salt.

The thyroid tissue was cooled to 5° C and transported to the laboratory within 10 to 30 minutes. Slices, 0.3 to 0.5 mm. thick, were prepared with a Stadie-Riggs microtome (Stadie and Riggs, 1944), gently blotted on filter paper, and rapidly weighed. The weight of each slice was about 100 mg. Alternate slices were fixed in formalin for histologic examination. The slices were incubated in 3 ml. of Krebs-Henseleit bicarbonate (Krebs and Henseleit, 1932) containing 0.2% of dextrose and 1 microcurie of carrier-free I^{131}. The I^{127} concentration of the medium was 0.2 μg. per 100 ml.[2]

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[1] Abridgment of portion of thesis submitted by Dr. Molnar to the Faculty of the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Medicine. (The Mayo Foundation, Rochester, Minnesota, is a part of the Graduate School of the University of Minnesota.)

[2] These determinations were carried out by the laboratory of Dr. M. H. Power by a modification of the method of Barker (1948).

Serial observations were made by measuring in a side arm of the incubating flask the changes in radioactivity of the medium. The side-arm incubating flask designed for this purpose was a siliconized, long-necked 50-ml. Erlenmeyer flask with a capillary tube 15 cm. long and 0.25 cm. in internal diameter connected to the bottom at an angle of 45 degrees. On the end of the capillary tube was a stainless-steel adapter to which a 2-ml. Luer-Lok syringe was attached. Each flask was fitted with an untapered rubber stopper. The rubber stopper was perforated by two glass tubes, one for inlet and the other for outlet of gas. Rubber tubes fitted on these glass tubes made possible airtight sealing of the flask with a single spring clamp. Lead shielding on top of the well-type scintillation counter served the dual purpose of shielding the flask and of permitting uniform positioning of the side-arm flasks.

Radioactivity was measured in the side arm by drawing the incubating fluid into it by the syringe. After determination of I^{131} (two counts with satisfactory statistical error), the medium was returned to the incubating flask by the syringe. It was thus possible to make repeated serial observations on the uptake of I^{131} by the tissue by measuring the disappearance of I^{131} from the medium, and to do so without opening or disrupting the incubating flask once it had been gassed with 95% oxygen and 5 per cent carbon dioxide. Except for the brief periods of measurement of I^{131}, the flasks were gently agitated in a water bath at 37° C. The amount of I^{131} remaining in the incubating medium was computed by reference to the I^{131} determination of the fluid made in identical fashion before the slice was added. This determination was taken as 100% for each flask. At the end of each experiment, the I^{131} in the rinsed and blotted slice was determined directly. Comparison of this value with a direct determination of an aliquot of the incubating fluid at the end of the experiment showed that the disappearance of I^{131} from the medium, as measured serially, accurately reflected its accumulation in the slice.

Protein-bound iodine (I^{127} and I^{131}) was determined by precipitation with trichloroacetic acid. The I^{127} content of the slice and of the medium after incubation was determined by Astwood and Bissell's modification (1944) of Kendall's method. A sodium iodide (thallium-activated) scintillation counter connected to a Model 1228 timer unit Multiscaler (Atomic Instrument Company) was used to determine I^{131} serially during incubation. A well-type Geiger counter (The Texas Company) was used to determine I^{131} during fractionation procedures on the slice after homogenization.

RESULTS

The pattern of I^{131} uptake as serially determined in slices of normal and hyperplastic human thyroids and of normal canine thyroids is shown in Figure 1. The general similarity in shape of these curves is evident. The shape of the curves indicated that the initial uptake in the first 30 minutes was much more rapid than the uptake subsequent to this interval. This raised the possibility that an inhibiting factor might be entering the medium to depress I^{131} uptake.

A crossover experiment to test this supposition was designed. Eight canine thyroid slices were prepared. Four of these slices were stored in the refrigerator. The remaining four slices were incubated immediately. After 280 minutes of incubation, two of the slices were removed and reincubated in standard fresh medium. The remaining two slices were not disturbed, and served as controls. At this same time the four stored and refrigerated slices were incubated, two in standard fresh medium and two in the medium from which the foregoing two slices had been removed at 280 minutes.

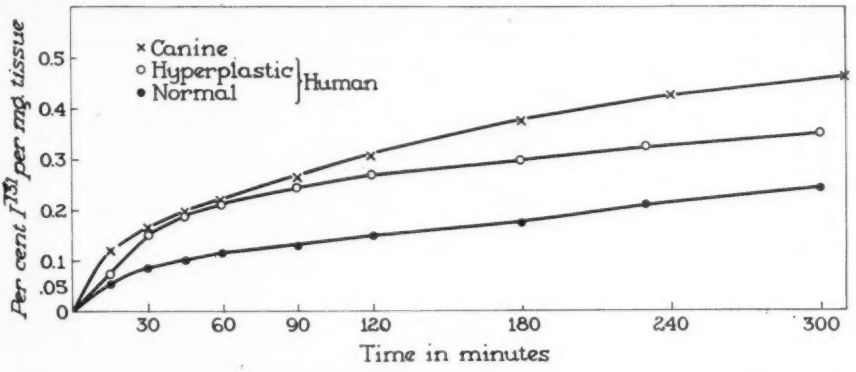

FIG. 1. Uptake of I^{131} by thyroid slices as per cent of dose per milligram wet weight of slice. Each dot represents the mean of 24 slices, each circle represents the mean of 12 slices and each cross represents the mean of 20 slices.

The results of this experiment are shown in Figure 2. Slices reincubated in standard fresh medium accumulated radioiodine again in a manner similar to the initial uptake, whereas the control slices maintained a more or less asymptotic level of I^{131}. Slices stored at 5° C for 280 minutes and incubated in standard fresh medium showed uptake curves similar to those of fresh tissue incubated immediately. Stored tissue accumulated less radioiodine when incubated in used medium from which fresh slices had been removed after 280 minutes of incubation than when incubated in

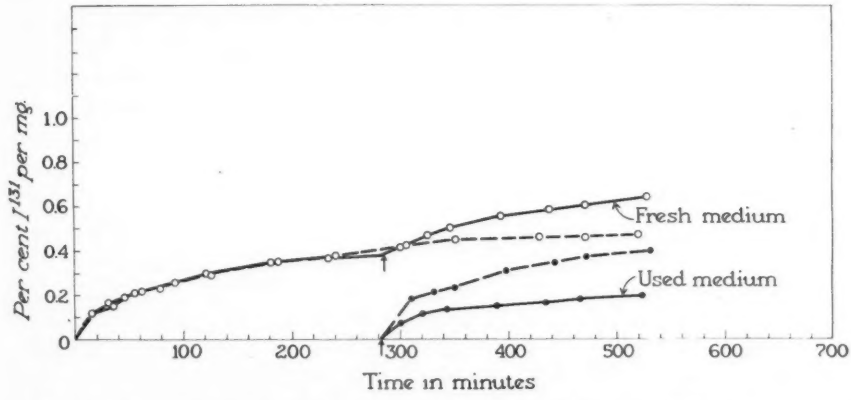

FIG. 2. Crossover experiment on alternate slices of the same thyroid. The upper curves (circles) represent slices incubated immediately. At 280 minutes, two slices were removed and reincubated in fresh medium. Note the immediate I^{131} uptake, as compared with the control. The lower curves (dots) represent alternate slices stored for 280 minutes and then incubated, some in the medium from which the foregoing slices had been removed and some in fresh medium. Note the difference in uptake of the slices in the "used" versus fresh medium.

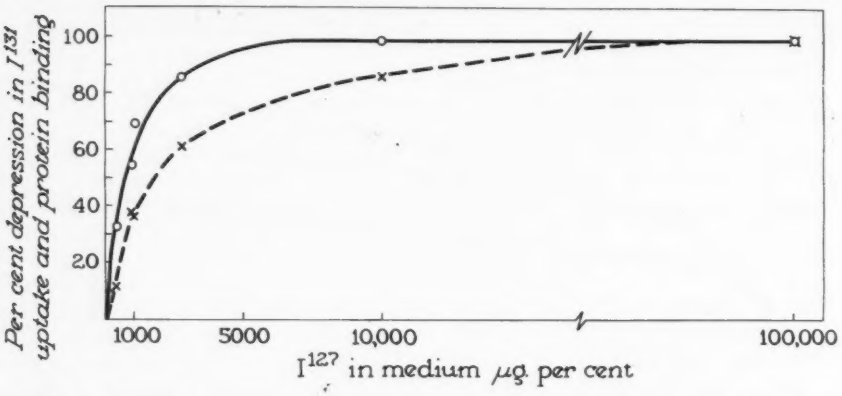

FIG. 3. Depression of I^{131} uptake (circles) and of protein binding (crosses) after 180 minutes of incubation at varying concentrations of I^{127} in medium. Each point was the average of two slices. Depression is expressed as per cent of uptake by control slices incubated in standard medium (0.2 μg. of I^{127} per 100 ml.).

fresh medium. Evidently, used medium contains materials inhibitory to I^{131} uptake.

To gain some information as to the nature of the inhibiting substances, the effect of diiodotyrosine, thyroxine, thyroglobulin[3] and sodium iodide on the pattern of I^{131} uptake in slices of canine thyroids was determined. These results are given in Figures 3 and 4. In confirmation of previous work, it was found that sodium iodide depressed the total uptake and the protein binding of I^{131} at concentrations greater than 150 micrograms per 100 ml. of medium. This value is in agreement with the findings (127 to 250 μg. of iodide per 100 ml. of medium) of Freinkel and Ingbar and of Fawcett and Kirkwood. In addition, organic iodine also depressed the uptake of radio-

TABLE 1. I^{127} CONTENT OF THE MEDIUM FOLLOWING INCUBATION OF HUMAN THYROID SLICES

Thyroid gland	No. of slices	Mean slice wt., mg.	Slice I^{127},* μg./mg.		Time of incubation, minutes	Medium I^{127}			Per cent I^{127} extracted from slice	
			Total	TCA† precipitable		Total‡ μg./100 ml.	Per cent TCA precipitable	Iodide increment, μg./100 ml.	Total	Iodide
1	6	100.3	0.46	0.42	300	900	78	198	59	156
2	2	88.7	0.78	0.67	60	592	100	0	26	0
	2	105.8			180	780	51	387	29	101
	2	108.3			300	855	55	386	30	98
	2	104.6			420	815	63	312	30	82
3	2	100.2	0.49	0.38	60	375	52	158	26	87
	2	92.0			180	530	74	148	39	73
	4	105.4			300	485	81	94	31	43

* As determined on alternate unincubated slices of the thyroid.
† TCA = trichloroacetic acid.
‡ The initial I^{127} content in each instance was 0.2 μg./100 ml.

[3] Thyroglobulin (Chilcott) CH-29, Lot #M5–43D. Total iodine 40.0 mg./100 ml., thyroxine iodine (Blau) 9.41 mg./100 ml. Kindly furnished by Dr. R. L. Kroc.

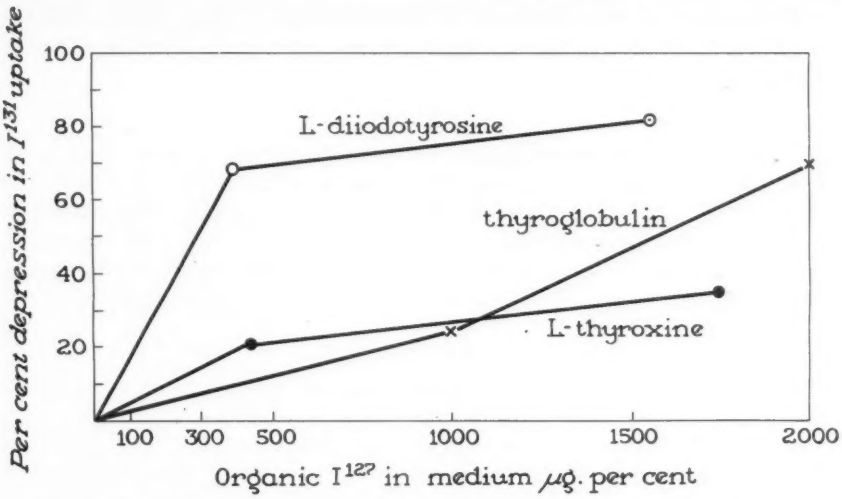

FIG. 4. Depression of I^{131} uptake after 180 minutes of incubation at varying concentrations of diiodotyrosine, thyroxine and thyroglobulin. Depression is expressed as per cent of uptake by control slices incubated in standard medium (0.2 μg. of I^{127} per 100 ml.).

iodine by the thyroid slices, although the amounts required were larger than that for inorganic iodide.

It remained to show that the medium in which slices were incubating contained iodide or organic iodine in quantities sufficient to depress the I^{131} uptake observed during incubation. Hence media were analyzed to determine both the amount and the nature of iodinated compounds. The table presents the analysis of media after exposure to thyroid slices for varying intervals. About one third of the total I^{127} of the slice, including practically all of its inorganic iodide, was extracted by the medium. Some 70% of the I^{127} in the medium was precipitable with trichloroacetic acid and was identified by paper chromatography as thyroglobulin. The remaining 30% of the iodine in the medium, which was soluble in trichloroacetic acid, behaved as iodide. The total I^{127} content of the medium after varying intervals of incubation was quite appreciable, ranging from 375 to 900 μg.[4] per 100 ml. of medium. This amount of iodine, whether organic or inorganic, was sufficient to depress the uptake of radioiodine by the slice. It appeared likely, therefore, that the depression of radioiodine uptake by the slice could be easily accounted for by the combined effect of the liberated organic and inorganic iodine.

[4] In eight of the 27 slices more iodide was present in the medium after incubation than was originally present in the slice. While this may be due in part to analytic error, it might also be accounted for by the presence of deiodinating systems liberating iodine from organic position (Roche and associates, 1951, and Tong and associates, 1954).

The authors wish to thank their surgical colleagues, Drs. Black, Beahrs, Judd and ReMine, for their interest and co-operation in obtaining human thyroid tissue.

SUMMARY

When slices of thyroid tissue were incubated in a fixed volume of medium, both organic and inorganic iodine were lost from the slice to the medium. The specific activity of the I^{131} in the medium was sufficiently lowered so that accumulation of radioiodine in the slice was depressed. Since the specific activity of the radioiodine is uncontrollable in the fixed-volume technic, studies made by this method are of limited significance.

REFERENCES

ASTWOOD, E. B. AND ADELE BISSELL: *Endocrinology* **34**: 282. 1944.
BARKER, S. B.: *J. Biol. Chem.* **173**: 715. 1948.
FAWCETT, D. M. AND S. KIRKWOOD: *J. Biol. Chem.* **204**: 787. 1953.
FREINKEL, N. AND S. H. INGBAR: *J. Clin. Endocrinol.* **15**: 442. 1955a.
FRIENKEL, N. AND S. H. INGBAR: *J. Clin. Endocrinol.* **15**: 598. 1955b.
KREBS, H. A. AND K. HENSELEIT: *Ztschr. Physiol. Chem.* **210**: 33. 1932.
MORTON, M. E. AND I. L. CHAIKOFF: *J. Biol. Chem.* **144**: 565. 1942.
MORTON, M. E. AND I. L. CHAIKOFF: *J. Biol. Chem.* **147**: 1. 1943.
MORTON, M. E., I. L. CHAIKOFF AND S. ROSENFELD: *J. Biol. Chem.* **154**: 381. 1944.
ROCHE, J., R. MICHEL, ODETTE MICHEL AND S. LISSITZKY: *Compt. rend. Soc. de biol.* **145**: 288. 1951.
SCHACHNER, H., A. L. FRANKLIN AND I. L. CHAIKOFF: *Endocrinology* **34**: 159. 1944.
STADIE, W. C. AND B. C. RIGGS: *J. Biol. Chem.* **154**: 687. 1944.
TONG, W., A. TAUROG AND I. L. CHAIKOFF: *J. Biol. Chem.* **207**: 59. 1954.

NOTES AND COMMENTS

ISOLATION OF AN UNKNOWN SUBSTANCE AND 6-KETOPROGESTERONE FROM PERFUSATES OF HUMAN PLACENTAE

The fact that progestational activity of blood in the umbilical artery is higher than in the vein suggested to Forbes (1955) that progesterone may be secreted by the fetus and removed by the placenta. We wish to report some pertinent results from our examinations of bovine blood perfusates of term or near-term human placentae obtained at Caesarian section.

A series of bovine blood perfusates of control and ACTH-stimulated placentae were individually extracted with isopropyl acetate and the extracts were partitioned between neohexane and 70% aqueous methanol. Adsorption chromatography of the methanolic extracts on silica gel was followed by partition chromatography on paper and finally by partition chromatography on columns of diatomaceous earth.

From those perfusions in which bovine blood was recycled through the placenta for no longer than one hour, a crystalline substance with the same mobility as progesterone on paper and column chromatograms was isolated. More of this substance was present in perfusates from ACTH-stimulated than from control placentae.

On paper chromatograms this substance gave an orange 2,4-dinitrophenyl-hydrazine reaction, an off-color Zimmerman test such as seen with a Δ^4-3-ketone, and a negative blue tetrazolium test (absence of an α-ketol group). Its chromatographic behavior on paper in the propylene glycol-toluene, propylene glycol-ligroin and cyclohexane-formamide systems was such that it could not be distinguished from authentic progesterone when run either side by side or as a mixed chromatogram. The substance crystallized as blades from hexane:ethyl acetate, 1:1; m.p. 149°–152°C.; $\alpha]_D + 139° \pm 23°$ in ethanol in a microcell;

$$\lambda^{max}_{methanol}\ 241\ m\mu; \qquad \lambda^{max}_{conc.\ H_2SO_4,\ 2\ hrs.}\ 294\ m\mu.$$

The infrared absorption spectrum of a solution in CS_2 in a microcell had two major bands, one at 1676 cm.$^{-1}$ (α,β-unsaturation) and the other at 1706 cm.$^{-1}$ (a hexacyclic or C-20 carbonyl). Unfortunately there was insufficient material for a close examination of the fingerprint region. In the Hooker-Forbes bioassay the substance was inactive in doses of 0.0032, 0.0064, 0.0096 and 0.0192 μg. Although the substance has a resemblance to progesterone, the melting point, possibly the optical rotation, and the inactivity in the Hooker-Forbes bioassay indicate that it is not progesterone. This substance was also isolated following a placental perfusion of progesterone and may therefore be a transformation product.

Progesterone-4-C^{14} (191 mg.; 4200 cts./min./mg.), purified by adsorption chromatography on silica gel and subsequent multiple recrystallizations, was, as a crystalline suspension in isotonic saline, metered into six liters of bovine blood just prior to its entrance into the placenta. The perfusate was not recycled through the organ but was collected immediately in a container chilled in an ice bath. The techniques of extraction and fractionation were the same as above.

A total of 103 mgs. of crystalline progesterone-4-C^{14} were recovered from the perfusate.

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66mgs. of a solid consisting of two different forms were crystallized from certain eluates of the column partition chromatogram. The two forms were mechanically separated and each was subjected to multiple recrystallizations. 50 mg. of progesterone-4-C^{14}, m.p. 129°–130° C, and about 1 mg. of the unknown substance, m.p. 154°–156° C., specific activity of 3070 cts./min./mg., were obtained. A mixed melting point of the two forms was 125°–131° C. The properties of the radioactive unknown were the same as those described above.

From this same perfusion 8 mg. of 6-ketoprogesterone (4080 cts./min./mg.) were also isolated. Identification was made by comparison with an authentic sample. The melting point (198°–200° C) absorption spectra in methanol and sulfuric acid, infrared spectrum and chromatographic behavior on paper were all identical. Although sought for, there was no evidence for the presence of detectable amounts of 6-hydroxylated progesterones.

In a control experiment in which progesterone-4-C^{14} in bovine blood was circulated through the perfusion apparatus in the absence of a placenta neither 6-ketoprogesterone nor the unknown substance could be detected.

Under the conditions of our experiment the conversion of progesterone to 6-ketoprogesterone and the unknown substance described above supports the suggestion by Forbes that term or near-term placenta may remove progesterone from the fetal blood circulation. Our data do not, however, reject the possibility that the placenta may synthesize progesterone.

Acknowledgments

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REFERENCE

Forbes, T. R.: *Endocrinology* **56**: 699. 1955.

TIME-RESPONSE EFFECT OF INSULIN UPON THE LEVEL OF PLASMA AMINO ACIDS IN THE FUNCTIONALLY EVISCERATED RAT

Insulin causes a decrease in the concentration of amino acids in the plasma of intact animals and suppresses the rate of rise in plasma amino acids following evisceration (see Russell, 1955, for review). The data of this report show that insulin causes a rapid but temporary decrease below normal in the level of plasma amino acids of functionally eviscerated rats.

METHODS

Male rats of 250 ±5 gm. were functionally eviscerated and given continuous intravenous infusions of saline or glucose (70 mg. per 100 gm. of rat per hour) with and without regular insulin (Lilly), at a dosage of 16 units per rat per 24 hours. The fluid load was 20 cc. per rat per 24 hours. The temperature was maintained at 26° ±0.5 C. At the end of the infusion period the level of plasma amino acids was determined on heparinized arterial blood by the method of Hamilton and Van Slyke (1943). (For details on methods see Ingle, Flores and Torralba, 1955.)

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EXPERIMENTS AND RESULTS

The data are in Table 1. In Experiment 1, 9 rats were eviscerated each day within a period of 30 minutes. One rat was bled immediately and the others were given continuous intravenous infusions of glucose and insulin. One rat was bled at each of the following intervals following evisceration: 15, 30, 45, 60, 90, 120, 180 and 240 minutes. This procedure was repeated each day with a random relationship between the order in which operations were completed and the length of the infusion period. The average values for plasma amino acids decreased significantly with time up to 60 minutes. After this series was completed, a second series was given the same glucose load without insulin

TABLE 1. LEVEL OF PLASMA AMINO ACIDS OF EVISCERATE RATS IN RELATION TO TIME AND TREATMENT; AVERAGES AND STANDARD ERROR FOR 15–16 RATS PER GROUP. MG. PER CENT

Experiment	Glucose load mg./100 gm./hr.	Insulin units/24/hr. per rat	Minutes following evisceration								
			0	15	30	45	60	90	120	180	240
1	70	16	5.01 ±0.236	4.27 ±0.214	3.92 ±0.271	3.39 ±0.212	3.35 ±0.186	3.47 ±0.188	3.55 ±0.219	3.44 ±0.218	3.86 ±0.219
	70	0	4.60 ±0.147	4.58 ±0.148	4.43 ±0.211	4.37 ±0.312	4.87 ±0.178				
2	70	16	4.62 0.194	4.13 ±0.180	3.85 ±0.218	3.52 ±0.271	2.98 ±0.185				
	70	0		4.77 ±0.129	4.43 0.218	4.62 ±0.226	4.82 ±0.225				
3	no infusion	0	4.69 ±0.16		4.87 ±0.14						
	saline only	0			4.52 ±0.127						
	70	0			4.73 ±0.161						
	70	16			3.60 ±0.132						

for periods of 15, 30, 45 and 60 minutes. Within this series the average values were unreliably lower than initial values at 30 and 45 minutes.

In Experiment 2, 9 eviscerated rats each day were given glucose with and without insulin for periods of 15, 30, 45 and 60 minutes. Comparisons of rats with and without insulin were made simultaneously. The average level of plasma amino acids decreased with time among the groups given insulin. Among the groups given glucose only, the average value 30 minutes following evisceration was unreliably lower than the average initial value.

In Experiment 3, the following conditions were represented on each day: no infusion, no delay; no infusion, 30 minute delay; saline infusion for 30 minutes; glucose infusion for 30 minutes; and, glucose and insulin infusion for 30 minutes. As in the other experiments there was a randomized order between the times that evisceration was completed and the subsequent treatment of the animal. Within this series only the rats given glucose and insulin showed a significantly lower level of plasma amino acids at 30 minutes following evisceration.

DISCUSSION

These data show that the administration of glucose with insulin to the eviscerate rat causes a temporary suppression in the level of plasma amino acids. It is doubtful that glucose alone or saline alone caused suppression in the level of plasma amino acids.

The suppression by insulin of the rise in plasma amino acids following evisceration has been thought to be due to a decrease in the rate of release of amino acids by the

tissues. The fact that insulin can, for a short period, suppress the level significantly below initial values is a basis for the suggestion that insulin may stimulate the synthesis of proteins under these conditions.

SUMMARY

When functionally eviscerated rats are given continuous intravenous infusions of glucose and insulin, there is a significant decrease in the level of plasma amino acids below initial values. There was no clearly established effect of infusions of glucose, or of saline without insulin.

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REFERENCES

HAMILTON, P. B. AND D. D. VAN SLYKE: *J. Biol. Chem.* **150**: 231. 1943.

INGLE, D. J., G. TORRALBA AND V. FLORES: *Proc. Soc. Exper. Biol. & Med.* **89**: 625–626. 1955.

RUSSELL, J. A.: *Fed. Proc.* **14**: 696. 1955.

COMPARISON OF A RADIOACTIVE ISOTOPE TECHNIQUE WITH TECHNIQUES USING FLAME PHOTOMETRY IN THE BIOASSAY OF MINERALOCORTICOIDS

The principal methods used for estimating the electrolyte-regulating activity of mineralocorticoids have been extensively reviewed by Dorfman (1954). They are based either on the counting of radioactive Na^{24} (or differential counting of Na^{24} and K^{42}) or on the estimation of ordinary Na and K by flame photometry, in the urine of rats to which a loading injection of the corresponding material has been previously administered. While Simpson and Tait (1952) have claimed that methods based on the counting of radioactive Na^{24} and K^{42} are preferable, no report has been hitherto published comparing the results of the two methods. Since facilities for handling radioactive isotopes are not always available however, it seemed important to determine whether simpler methods based on flame photometry were reliable. Since this investigation was undertaken with the sole object of assessing the reliability of the methods of assay, desoxycorticosterone acetate (DCA) was the only steroid used for this comparative evaluation.

METHODS

The biological system consisted of young white rats of an average weight of 75 gm. bred in our departments. After adrenalectomy the rats were fed a standard diet (Llaurado, 1955c.) of synthetic pellets and given isotonic saline *ad lib.* to drink. Assays were carried out on the 4th and 6th day after operation. Each group of test animals consisted of 7 rats, and a control group of the same size was used on each day of testing. In preliminary experiments it was observed that variations between control groups from one session to another were greater than the differences between males and females. For this reason both male and female rats were used in these experiments, though all animals in any one assay were of the same sex. On rare occasions one or more rats in either the experimental or the control groups died or showed extreme weakness at the end of the bioasay, and values for these animals were discarded. In each session 4 or 5 groups were used. At 9 A.M. on the morning of the bioassay food and drinking fluid were withdrawn from the cages. Subcutaneous injections of steriod dissolved in 0.1 ml. of 20% ethanol in water were made at 10 A.M. and at the same time the bladders of all rats were emptied by applying gentle suprapubic pressure combined with a light inhalation of ether. From this stage onwards the methods followed different paths requiring separate description.

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Method I (M-I).—At 11 A.M. a subcutaneous injection of 1 ml. of distilled water containing a mixture of 73 μg. of Na^{24} and 295 μg. of K^{42} was given, the total radioactivity being about 60 μc. Immediately after injection each rat was placed in a separate metabolism cage which had been scrupulously cleaned. At 2 P.M. the rats were made to urinate by the method described above, all urine being collected in the metabolism cages. These were carefully rinsed with 5 ml. of distilled water projected against the walls of the cages by means of a syringe and needle. The diluted urines were collected in small bottles. The ratio between Na^{24} and K^{42} counts was determined in each sample by a modification (Cope and Llaurado, 1954) of the method described by Tait and Williams (1952). The degree of activity of the electrolyte-regulating substance assayed was indicated by the fall in the ratio of Na^{24} to K^{42} in the urine samples, and the mean value of this ratio for the experimental animals was expressed as a percentage of the mean value obtained for the control group on the same session.

Method II (M-II).—The remainder of each urine sample collected in M-I was stored in the refrigerator for at least fourteen days before estimations of total Na and K were made. These were carried out following a procedure designed to estimate a large number of samples in a short time (Cardus and Llaurado, 1955) using an EEL[1] flame photometer. The ratio of Na to K in the urine was calculated and the results were again expressed as a percentage of the mean figure obtained for the control at the same time.

Method III (M-III).—The rats used in the previous experiments were kept alive with isotonic saline for drinking fluid and used again two days later[2] for a similar assay, but in this case a load of 1 mg. Na and 10 mg. K contained in 1 ml. of distilled water was injected into each rat. Apart from this, the assay followed the same stages as described in M-I. At the end of the urine collection, the metabolism cages were rinsed with 10 ml. of distilled water. The flame photometer was also used for the Na and K estimations.

RESULTS

Four different doses of DCA were assayed by M-I and M-II , and 5 different doses by M-III. The mean values for each dose level of DCA obtained by each of the three methods of assay, with the corresponding standard errors, are shown in Table 1. Regression lines for each method were calculated by the method of the least squares (Table 1).

TABLE 1. COMPARATIVE DATA OF BIOASSAYS WITH DCA

Dose, μg.	M-I		M-II		M-III	
	k*	m ± S.E.†	k*	m ± S.E.†	k*	m ± S.E.†
5	3	99.93 ± 16.07	3	109.19 ± 19.77	4	75.37 ± 9.26
7	—	—	—	—	3	56.32 ± 10.17
10	12	51.69 ± 4.62	12	46.91 ± 4.36	10	50.54 ± 4.06
14	1	42.80 ± 18.13	1	45.53 ± 22.26	—	—
20	1	15.67 ± 8.73	1	22.80 ± 11.73	1	37.14 ± 7.95
25	—	—	—	—	2	25.80 ± 5.46
Regression line	$y+140.27x=194.16$		$y+153.91x=206.51$		$y+63.30x=115.14$	
S.D.L.‡	5.77		12.67		4.38	
S.E.$_b$§	18.92		41.54		9.54	
$L=\frac{1}{\lambda}=\frac{\lvert b\rvert}{\text{S.D.L.}}$	24.39		12.20		14.49	

* No. of bioassays.

† Mean ± S.E.—Standard error calculated as in Llaurado (1955a).

‡ $\text{S.D.L.}=\sqrt{\frac{\Sigma(y-y\ \text{calc.})^2}{N-2}}$ = Standard deviation about the line.

§ $\text{S.E.}_b=\frac{\text{S.D.L.}}{\sqrt{\Sigma(x-\bar{x})^2}}$ = standard error of the slope.

It was found that there was a linear relationship between the logarithm of the dose of DCA and $(100\times R_D)/R_C$, where R_D is the mean ratio of Na/K in the treated animals and R_C the corresponding value in the controls.

[1] Evans Electroselenium Limited, Harlow, Essex, U.K.

[2] These animals were subdivided into groups for this bioassay by a process of randomization to minimize the possibility of error due to cumulative effect of the steroid.

DISCUSSION

The slopes of the regression lines (Table 1) for M-I and M-II obtained from results on the same samples by these two methods do not differ significantly. On the other hand, the slopes between M-III and either of the other two methods differ significantly ($P < 0.001$). The *index of precision* (L) gave good values (Table 1) for all methods.

As far as the sensitivity of the assay is concerned, this seemed to depend on the amount of electrolyte injected into the rat, being distinctly greater in M-III in which the amount of 1 mg. Na and 10 mg. K administered must influence the distribution of the electrolytes in the body of the rat, while the quantities of radioactive material injected are practically negligible from this point of view. This observation is consistent with the finding by Dorfman (1949) that better results are obtained in this type of bioassay when a high load of K is given.

The conclusion reached from this comparative study is that for assay of electrolyte-regulating corticoids methods employing flame photometry without use of radioactive isotopes are as reliable as those in which such isotopes are used. An advantage of the flame photometry methods is that the samples can be stored until it is convenient to carry out the analyses. In previous work (Cope and Llaurado, 1954) M-I was used for the estimation of the electrolyte-regulating corticoid present in human urine. In recent times it has been found convenient, in using M-III, to reduce the interval between adrenalectomy and bioassay from six to four days and it has been shown that this modification does not significantly alter the results obtained. The method thus modified has been used for the detection of the electrolyte-regulating corticoid (presumably aldosterone) in urine after operation (Llaurado, 1955a) and for the pharmacological estimation of aldosterone and 9α-halocortisols (Llaurado, 1955b).

SUMMARY

A method for the bioassay of mineralocorticoids based on the use of radioactive Na^{24} and K^{42} has been compared with two other methods in which radioactive isotopes are not used, and Na and K are estimated by the flame photometer. From the results obtained it is concluded that the three methods are equally reliable and that in consequence the use of radioactive materials does not represent any real advantage.

Acknowledgments

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REFERENCES

Cardus, D. and J. G. Llaurado: *Med. clin.* **24**: 193. 1955.
Cope, C. L. and J. G. Llaurado: *Brit. med. J.* **1**: 1290. 1954.
Dorfman, R. I.: *Proc. Soc. exp. Biol., N. Y.* **70**: 732. 1949.
Dorfman, R. I.: *Physiol. Rev.* **34**: 138. 1954.
Llaurado, J. G.: *Lancet* **1**: 1295. 1955a.
Llaurado, J. G.: *Experientia* **11**: 401. 1955b.
Llaurado, J. G.: *Endocrinology* **57**: 516. 1955c.
Simpson, S. A. and J. F. Tait: *Endocrinology* **50**: 150. 1952.
Tait, J. F. and E. S. Williams: *Nucleonics* **10**: 47. 1952.

THE ENDOCRINE SOCIETY

THE EIGHTH POSTGRADUATE ASSEMBLY IN ENDOCRINOLOGY AND METABOLISM

Sponsored jointly by the Endocrine Society; The University of Texas Postgraduate School of Medicine, and the University of Texas M. D. Anderson Hospital and Tumor Institute,
Houston, Texas.
October 2–27, 1956

The faculty will consist of twenty-two eminent clinicians and investigators from various parts of the country in the fields of endocrinology and metabolism. The program will cover the various endocrinopathies, with emphasis on the clinical aspects, demonstration of laboratory tests, presentations of cases, and question-and-answer panel discussions. The course is designed to cover the main aspects of diagnosis and therapy in the field of endocrinology and metabolism for the physician in general practice and for those in other specialties who wish to have a general knowledge of this rapidly growing field.

A syllabus with brief abstracts of lectures will be available to the registrants at the time of the assembly.

For further information concerning the program and registration, write to Dr. J. B. Trunnel, M. D. Anderson Hospital, 6723 Bertner Avenue, Houston 25, Texas. Registration is limited to 100; tuition fee is $100.00. Rooms will be reserved for the students and faculty at the Shamrock Hilton Hotel which is adjacent to the Texas Medical Center.

THE INTERNATIONAL FERTILITY ASSOCIATION

Regional meeting of the United States division of the International Fertility Association will be held at the Schenley Park Hotel, Pittsburgh, Pa., on March 14, 1956. Interesting scientific papers on infertility will be presented; also panel discussion. All physicians are invited. For further information write to Maxwell Roland, M.D., Chairman, Liaison Committee, 1141 Eastern Parkway, Brooklyn, N. Y.

GORDON RESEARCH CONFERENCE

The Gordon Research Conference on Chemistry, Physiology and Structure of Bones and Teeth sponsored by the American Association for the Advancement of Science for 1956 will be held July 16 to 20 at Kimball Union Academy, Meriden, New Hampshire.

Individuals interested in attending the Conference are requested to send in their applications to the Director. Each applicant must state the institution or company with which he is connected and the type of work in which he is most interested. Attendance at each Conference is limited to 100.

Requests for attendance at the Conference, or for any additional information should be addressed to W. George Parks, Director, Department of Chemistry, University of Rhode Island, Kingston, Rhode Island. From June 11 to August 31, 1956 mail should be addressed to Colby Junior College, New London, New Hampshire.

CHEMISTRY, PHYSIOLOGY AND STRUCTURE OF BONES AND TEETH

R. F. SOGNNAES, Chairman, D. H. COPP, Vice Chairman

16 July:

G. WISLOCKI, Chairman. Ground Substance.
K. MEYER. Chemical Nature of Ground Substance.
L. BELANGER. Histochemical Distribution of Ground Substance in Bones and Teeth.
D. W. FAWCETT, Chairman, S. GLASSTONE-HUGHES. Experimental Studies in Tooth Development.

17 July:

W. NEUMAN, Chairman. The Skeleton as an Alkaline Reserve.
W. STOLL. Chemical Studies on the Nature of Bone Sodium.
G. B. FORBES. The Contribution of the Skeleton in Sodium Homeostasis.
R. BOGOROCH, P. YEN, J. H. SHAW and R. F. SOGNNAES. Comparative Studies of Radiosodium Turnover in Bones and Teeth of Rats and Rhesus Monkeys.
W. D. ARMSTRONG, Chairman, Y. ERICSSON. Solubility of Calcium Phosphate with Special Reference to the Liquid-Solid System of Saliva, Enamel and Tartar Deposition.

18 July:

M. Logan, Chairman. Citric Acid and Vitamin D.
L. Singer and W. D. Armstrong. *In Vitro* Studies on Uptake and Turnover of Citrate in Calcified Tissues.
M. S. Thompson and A. B. Hastings. Factors Affecting the Citrate Content of Hydroxylapatite.
J. Crawford, D. Gribetz, W. Canada, P. Hurst and B. Castelman. Influence of Vitamin D on Calcium and Citrate Metabolism in Rats.
P. E. Boyle, Chairman.
J. T. Irving. A Comparison of the Influences of Various Dietary Factors upon the Calcification Sequences in Enamel, Dentin and Bone.

19 July:

R. Harris, Chairman. Bone Seekers.
J. C. Aub and W. B. Looney. Long-Term Observations Following Administration of Radium, Lead and Thorium.
J. Jowsey. Mechanism of Yttrium, Strontium and Sulfur Deposition in Bone.
H. Foreman. Mode of Plutonium and Strontium Combination with Bone.
F. C. Mclean, Chairman.
B. Engfeldt. Bone and Tooth Pathology Investigated by Microradiography and Microinterferometry.

20 July:

H. Hodge, Chairman. Panel Discussion on the Mechanism of Calcification.
R. S. Bear. Structure of Collagen.
W. F. Neuman. The Earliest Events in Calcification.
R. A. Robinson. Crystal-Water Replacement During the Calcification of Osteoid.
A. E. Sobel. The Initiation of Calcification.

EASTERN PSYCHIATRIC RESEARCH ASSOCIATION

This is the name of a newly organized association of psychiatrists chartered in New York State. The first President is David Impastato, M.D., of New York, N. Y. Vice President is Leo Alexander, M.D., of Boston, and Theodore R. Robie, M.D., of East Orange, is Secretary-Treasurer.

The protocol of this new organization's aims and purposes includes the following: "To initiate, encourage and foster research in psychiatry and related fields of medicine . . . to stimulate scientific study and research into the nature and development of human behavior; to organize and coordinate public and private research and efforts in the application of sound principles of psychiatric knowledge . . . to encourage the exchange of scientific knowledge and information in all fields of medicine and especially in the field of psychiatry and mental hygiene."

The Association will hold four meetings per year, including the annual meeting in October. At that meeting, an incentive prize will be awarded for the presentation of the best scientific report.

For further information, address Theodore R. Robie, M.D., Secy.-Treas., 676 Park Avenue, East Orange, N. J. Official transactions will be published in "Diseases of the Nervous System."

1956 LAURENTIAN HORMONE CONFERENCE

The 1956 Laurentian Hormone Conference of the AAAS will be held at Mont Tremblant Lodge, Mont Tremblant, Quebec, during the period September 2–7, and investigators interested in attending this conference should make application to the Committee on Arrangements of The Laurentian Hormone Conference, 222 Maple Avenue, Shrewsbury, Massachusetts, at an early date and in any event no later than May 7, 1956. A conference rate of $12.00 per person per day is extended to all invited participants. Attendance is limited to invitations issued by the Committee on Arrangements. The following program has been arranged.

I. *Neurohumoral-Endocrine Relationships*

Biochemical, physiological and pharmacological aspects of serotonin.

S. Udenfriend, P. A. Shore, C. F. Bogdanski and B. B. Brodie, National Institutes of Health.

Sunday evening, September 2

Endocrine function following transections of upper brain stem and spinal cord.

E. Anderson, R. W. Bates, E. Hawthorne, W. Haymaker, D. McK. Rioch, W. T. Spence, and H. Wilson, National Institutes of Health.

Monday morning, September 3

The influence of the central nervous system on pituitary function.

Monte Greer, Veterans Administration Hospital, Long Beach, California

Monday morning, September 3

Hormones and rhythms.

Curt P. Richter, Johns Hopkins Hospital, Baltimore, Maryland.

Monday evening, September 3

II. *Hormone Transport in Circulation*

The interaction of thyroid hormones and protein in biological fluids.

J. E. Rall and Jacob Robbins, National Institutes of Health.

Tuesday morning, September 4

The binding of steroids and their conjugates to human plasma proteins.

A. A. Sandberg and W. R. Slaunwhite, Jr., Roswell Park Memorial Institute.

Tuesday morning, September 4

III. *Aspects of Reproduction*

Some experimental studies on the mechanism of mammalian ova implantation.

M. C. Shelesnyak, The Weizmann Institute of Science, Israel.
Tuesday evening, September 4

Progestines in the normal menstrual cycle.

John Rock, Free Hospital for Women, Brookline.
Wednesday morning, September 5

The metabolism of progesterone and its clinical use.

M. E. Davis, University of Chicago Medical School.
Wednesday morning, September 5

Long-acting steroids in reproductive processes.

Karl Junkmann, Schering, A. G., West Berlin, Germany.
Thursday morning, September 6

IV. *Hormone Chemistry and Metabolism*

Glucagon, a second pancreatic hormone.

P. P. Foa, The Chicago Medical School.
Thursday morning, September 6

Insulinase and insulinase inhibitors.

I. Arthur Mirsky, University of Pittsburgh.
Thursday evening, September 6

V. *Hormones and Stress*

Endocrine responses in surgery.

Francis Moore, Peter Bent Brigham Hospital, Boston.
Friday morning, September 7

Adrenal influences upon stomach and gastric responses to stress.

Seymour J. Gray, Peter Bent Brigham Hospital, Boston.
Friday morning, September 7